m-96 B2

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/12, 15/62, 15/67, 1/19, 1/21, 5/10, A61K 38/16, 39/395, 48/00, C07K 14/435, 14/715, 16/18, 16/28, 19/00, G01N 33/53, 37 /00

A1

(11) International Publication Number: **WO 95/31544**

(43) International Publication Date: 23 November 1995 (23.11.95)

(21) International Application Number: PCT/US95/05854

(22) International Filing Date: 11 May 1995 (11.05.95)

(30) Priority Data:

109632	11 May 1994 (11.05.94)	IL
111125	2 October 1994 (02.10.94)	IL

(71) Applicant *(for all designated States except US)*: YEDA RESEARCH AND DEVELOPMENT CO., LTD. [IL/IL]; At the Weizmann Institute of Science, P.O. Box 95, 76100 Rehovot (IL).

(71) Applicant *(for VN only)*: WEINWURZEL, Henry [US/IL]; Herzl Street 43, 43353 Raanana (IL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: WALLACH, David [IL/IL]; 24 Borochov Street, 76406 Rehovot (IL). BOLDIN, Mark [RU/RU]; Zelenograd, 927-53, Moscow, 103575 (RU). METT, Igor [IL/IL]; 60 Levin Epstein Street, 76462 Rehovot (IL). VARFOLOMEEV, Eugene [IL/IL]; Dept. of Membrane Research and Biophysics, Weizmann Institute of Science, P.O. Box 26, 76100 Rehovot (IL).

(74) Agent: BROWDY, Roger, L.; Browdy and Neimark, 419 Seventh Street, N.W., #300, Washington, DC 20004 (US).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.

(54) Title: MODULATOR OF TNF/NGF SUPERFAMILY RECEPTORS AND SOLUBLE OLIGOMERIC TNF/NGF SUPERFAMILY RECEPTORS

(57) Abstract

The present invention generally concerns novel proteins which bind to the intracellular domains of the p55 and p75 TNF-Rs and the Fas-R, which are capable of modulating the function of the p55 and p75 TNF-Rs and the Fas-R, and the DNA sequences which encode them. The present invention also concerns new soluble oligomeric TNF-Rs, oligomeric Fas-Rs and oligomeric receptors having a mixture of TNF-Rs and Fas-Rs. In addition, the present invention concerns methods of preparation and uses of all of the aforementioned.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

MODULATOR OF TNF/NGF SUPERFAMILY RECEPTORS AND SOLUBLE OLIGOMERIC TNF/NGF SUPERFAMILY RECEPTORS

Field of the Invention

The present invention is generally in the field of receptors belonging to the TNF/NGF superfamily of receptors and the control of their biological functions. The TNF/NGF superfamily of receptors includes receptors such as the p55 and p75 tumor necrosis factor receptors (TNF-Rs) and the FAS ligand receptor (also called FAS/APO1 or FAS-R and hereinafter will be called FAS-R) and others. More specifically, the present invention concerns novel proteins which bind to the intracellular domains (IC) of the p55 and p75 TNF-Rs and the Fas-R, (these intracellular domains designated p55IC, p75IC and Fas-IC, respectively) and which novel proteins are capable of modulating the function of the p55 and p75 TNF-Rs and the Fas-R. One of the proteins capable of binding the p55IC of the imact p55-TNF-R is the p55IC itself in the form of a p55IC molecule or a portion thereof, such as for example. the so-called 'death domain' (DD) of the p55IC. Thus, the present invention also concerns new TNF-associated effects that can be induced in cells in a ligand (TNF)-independent fashion by the intracellular domain of the p55 TNF-R (p55IC) or portions thereof. The present invention also concerns the preparation and uses of these novel p55 and p75 TNF-R-binding proteins, and Fas-R binding proteins, referred to herein as p55IC-, p75IC- and Fas-IC- binding proteins.

In another aspect, the present invention also concerns new soluble oligomeric TNF-Rs, oligomeric FAS-Rs and oligomeric receptors having a mixture of TNF-Rs and FAS-Rs, their uses, and methods for the production thereof.

Background of the Invention and Prior Art

Tumor Necrosis Factor (TNF-α) and Lymphotoxin (TNF-β) (hereinafter, TNF, refers to both TNF-α and TNF-β) are multifunctional pro-inflammatory cytokines formed mainly by mononuclear phagocytes, which have many effects on cells (Wallach, D. (1986) in : Interferon 7 (Ion Gresser, ed.), pp. 83-122, Academic Press, London; and Beutler and Cerami (1987)). Both TNF-α and TNF-β initiate their effects by binding to specific cell surface receptors. Some of the effects are likely to be beneficial to the organism : they may destroy, for example tumor cells or virus infected cells and augment antibacterial activities of granulocytes. In this way, TNF contributes to the defense of the organism against tumors and infectious agents and contributes to the recovery from injury. Thus, TNF can be used as an anti-tumor agent in which application it binds to its receptors on the surface of tumor cells and thereby initiates the events leading to the death of the tumor cells. TNF can also be used as an anti-infectious agent.

However, both TNF-α and TNF-β also have deleterious effects. There is evidence that over-production of TNF-α can play a major pathogenic role in several diseases. Thus, effects of TNF-α, primarily on the vasculature, are now known to be a major cause for symptoms of septic shock (Tracey et al., 1986). In some diseases, TNF may cause excessive loss of weight (cachexia) by suppressing activities of adipocytes and by causing anorexia, and TNF-α was thus called cachetin. It was also described as a mediator of the damage to tissues in rheumatic diseases (Beutler and Cerami, 1987) and as a major mediator of the damage observed in graft-versus-host reactions (Piquet et al., 1987). In addition, TNF is known to be involved in the process of inflammation and in many other diseases.

Two distinct, independently expressed, receptors, the p55 and p75 TNF-Rs, which bind both TNF-α and TNF-β specifically, initiate and/or mediate the above noted biological effects of TNF. These two receptors have structurally dissimilar intracellular domains suggesting that they signal differently (See Hohmann et al., 1989; Engelmann et al., 1990; Brockhaus et al., 1990; Leotscher et al., 1990. Schall et al., 1990; Nophar et al., 1990; Smith et al., 1990; and Heller et al., 1990). However, the cellular mechanisms, for example, the various proteins and possibly other factors, which are involved in the intracellular signaling of the p55 an p75 TNF-Rs have yet to be elucidated (as set forth herein below, there is described for the first time, new proteins capable of binding to the p75IC and p55 IC). It is this intracellular signaling, which occurs usually after the binding of the ligand, i.e. TNF (α or β), to the receptor, that is responsible for the commencement of the cascade of reactions that ultimately result in the observed response of the cell to TNF.

As regards the above mentioned cytocidal effect of TNF, in most cells studied so far, this effect is triggered mainly by the p55 TNF-R. Antibodies against the extracellular domain (ligand binding domain) of the p55 TNF-R can themselves trigger the cytocidal effect (see EP 412486) which correlates with the effectivity of receptor cross-linking by the antibodies, believed to be the first step in the generation of the intracellular signaling process. Further, mutational studies (Brakebusch et al., 1992; Tartaglia et al., 1993) have shown that the biological function of the p55 TNF-R depends on the integrity of its intracellular domain, and accordingly it has been suggested that the initiation of intracellular signaling leading to the cytocidal effect of TNF occurs as a consequence of the association of two or more intracellular domains of the p55 TNF-R. Moreover, TNF (α and β) occurs as a homotrimer and as such has been suggested to induce intracellular signaling via the p55 TNF-R by way of its ability to bind to and to cross-link the receptor molecules, i.e. cause receptor aggregation. Herein below there is described how the p55IC and p55DD can self-associate and induce, in a ligand-independent fashion, TNF-associated effects in cells.

Another member of the TNF/NGF superfamily of receptors is the FAS receptor (FAS-R) which has also been called the Fas antigen, a cell-surface protein expressed in various tissues and sharing homology with a number of cell-surface receptors including TNF-R and NGF-R. The FAS-R mediates cell death in the form of apoptosis (Itoh et al., 1991), and appears to serve as a negative selector of autoreactive T cells, i.e. during maturation of T cells, FAS-R mediates the apoptopic death of T cells recognizing self-antigens. It has also been found that mutations in the

FAS-R gene (*lpr*) cause a lymphoproliferation disorder in mice that resembles the human autoimmune disease systemic lupus erythematosus (SLE) (Watanabe-Fukunaga et al., 1992). The ligand for the FAS-R appears to be a cell-surface associated molecule carried by, amongst others, killer T cells (or cytotoxic T lymphocytes - CTLs), and hence when such CTLs contact cells carrying FAS-R, they are capable of inducing apoptopic cell death of the FAS-R-carrying cells. Further, a monoclonal antibody has been prepared that is specific for FAS-R, this monoclonal antibody being capable of inducing apoptopic cell death in cells carrying FAS-R, including mouse cells transformed by cDNA encoding human FAS-R (Itoh et al., 1991).

It has also been found that various other normal cells, besides T lymphocytes, express the FAS-R on their surface and can be killed by the triggering of this receptor. Uncontrolled induction of such a killing process is suspected to contribute to tissue damage in certain diseases, for example, the destruction of liver cells in acute hepatitis. Accordingly, finding ways to restrain the cytotoxic activity of FAS-R may have therapeutic potential.

Conversely, since it has also been found that certain malignant cells and HIV-infected cells carry the FAS-R on their surface, antibodies against FAS-R, or the FAS-R ligand, may be used to trigger the FAS-R mediated cytotoxic effects in these and thereby provide a means for combating such malignant cells or HIV-infected cells (see Itoh et al., 1991). Finding yet other ways for enhancing the cytotoxic activity of FAS-R may therefore also have therapeutic potential.

It has been a long felt need to provide a way for modulating the cellular response to TNF (α or β) and FAS-R ligand, for example, in pathological situations as mentioned above, where TNF or FAS-R ligand is over-expressed it is desirable to inhibit the TNF- or FAS-R ligand-induced cytocidal effects, while in other situations, e.g. wound healing applications, it is desirable to enhance the TNF effect, or in the case of FAS-R, in tumor cells or HIV-infected cells it is desirable to enhance the FAS-R mediated effect.

A number of approaches have been made by the present inventors (see for example, European Application Nos. EP 186833, EP 308378, EP 398327 and EP 412486) to regulate the delererious effects of TNF by inhibiting the binding of TNF to its receptors using anti-TNF antibodies or by using soluble TNF receptors (being essentially the soluble extracellular domains of the receptors) to compete with the binding of TNF to the cell surface-bound TNF-Rs. Further, on the basis that TNF-binding to its receptors is required for the TNF-induced cellular effects, approaches by the present inventors (see for example EPO 568925) have been made to modulate the TNF effect by modulating the activity of the TNF-Rs. Briefly, EPO 568925 relates to a method of modulating signal transduction and/or cleavage in TNF-Rs whereby peptides or other molecules may interact either with the receptor itself or with effector proteins interacting with the receptor, thus modulating the normal functioning of the TNF-Rs. In EPO 568925 there is described the construction and characterization of various mutant p55 TNF-Rs, having mutations in the extracellular, transmembranal, and intracellular domains of the p55 TNF-R. In this way regions within the above domains of the p55 TNF-R were identified as being essential to the functioning of the receptor, i.e. the binding of the ligand (TNF) and the subsequent signal transduction and intracellular signaling which ultimately results in the observed TNF-effect on the

cells. Further, there is also described a number of approaches to isolate and identify proteins, peptides or other factors which are capable of binding to the various regions in the above domains of the TNF-R, which proteins, peptides and other factors may be involved in regulating or modulating the activity of the TNF-R. A number of approaches for isolating and cloning the DNA sequences encoding such proteins and peptides; for constructing expression vectors for the production of these proteins and peptides; and for the preparation of antibodies or fragments thereof which interact with the TNF-R or with the above proteins and peptides that bind various regions of the TNF-R, are also set forth in EPO 568925. However, no description is made in EPO 568925 of the actual proteins and peptides which bind to the intracellular domains of the TNF-Rs (e.g. p55 TNF-R), nor is any description made of the yeast two-hybrid approach to isolate and identify such proteins or peptides which bind to the intracellular domains of TNF-Rs. Similarly, heretofore there has been no disclosure of proteins or peptides capable of binding the intracellular domain of FAS-R.

Thus, when it is desired to inhibit the effect of TNF, or the FAS-R ligand, it would be desirable to decrease the amount or the activity of TNF-Rs or FAS-R at the cell surface, while an increase in the amount or the activity of TNF-Rs or FAS-R would be desired when an enhanced TNF or FAS-R ligand effect is sought. To this end the promoters of both the p55 TNF-R and the p75 TNF-R have recently been sequenced and analyzed by the present inventors and a number of key sequence motifs have been found that are specific to various transcription regulating factors, and as such the expression of these TNF-Rs can be controlled at their promoter level, i.e. inhibition of transcription from the promoters for a decrease in the number of receptors, and an enhancement of transcription from the promoters for an increase in the number of receptors (see IL 104355 and IL 109633 and their corresponding, as yet unpublished EP and PCT counterparts). Corresponding studies concerning the control of FAS-R at the level of the promoter of the FAS-R gene have yet to be reported.

Further, it should also be mentioned that, while it is known that the tumor necrosis factor (TNF) receptors, and the structurally-related receptor FAS-R, trigger in cells, upon stimulation by leukocyte-produced ligands, destructive activities that lead to their own demise, the mechanisms of this triggering are still little understood. Mutational studies indicate that in FAS-R and the p55 TNF receptor (p55-R) signaling for cytotoxicity involve distinct regions within their intracellular domains (Brakebusch et al., 1992; Tartaglia et al., 1993; Itoh and Nagata, 1993). These regions (the 'death domains') have sequence similarity. The 'death domains' of both FAS-R and p55-R tend to self-associate. Their self-association apparently promotes that receptor aggregation which is necessary for initiation of signaling (as set forth herein below, as well as Song et al., 1994; Wallach et al., 1994; Boldin et al., 1995) and at high levels of receptor expression can result in triggering of ligand-independent signaling (as set forth herein below, and Boldin et al., 1995).

Thus, prior to the present invention, there have not been provided proteins which may regulate the effect of ligands belonging to the TNF/NGF superfamily, such as the TNF or FAS-R ligand effect on cells, by mediation of the intracellular signaling process, which signaling is probably governed to a large extent by the intracellular domains (ICs) of the receptors belonging

to the TNF/NGF superfamily of receptors, such as those of the TNF-Rs, i e. the p55 and p75 TNF-R intracellular domains (p55IC and p75IC, respectively), as well as the FAS-IC.

Accordingly, it is one aim of the invention to provide proteins which are capable of binding to the intracellular domains of the TNF-Rs and FAS-R, which proteins are presently believed to be involved in the intracellular signaling process initiated by the binding of TNF to its receptors, or the binding of FAS ligand to its receptor.

Another aim of the invention is to provide antagonists (e.g. antibodies) to these intracellular domain-binding proteins (IC-binding proteins) which may be used to inhibit the signaling process, when desired, when such IC-binding proteins are positive signal effectors (i.e. induce signaling), or to enhance the signaling process, when desired, when such IC-binding proteins are negative signal effectors (i.e. inhibit signaling).

Yet another aim of the invention is to use such IC-binding proteins to isolate and characterize additional proteins or factors, which may, for example, be involved further downstream in the signaling process, and/or to isolate and identify other receptors further upstream in the signaling process to which these IC-binding proteins bind (e.g. other TNF-Rs or related receptors), and hence, in whose function the IC-binding proteins are also involved.

Moreover, it is an aim of the present invention to use the above-mentioned IC-binding proteins as antigens for the preparation of polyclonal and/or monoclonal antibodies thereto. The antibodies, in turn, may be used for the purification of the new IC-binding proteins from different sources, such as cell extracts or transformed cell lines.

Furthermore, these antibodies may be used for diagnostic purposes, e.g. for identifying disorders related to abnormal functioning of cellular effects mediated by receptors belonging to the TNF/NGF receptor superfamily.

A further aim of the invention is to provide pharmaceutical compositions comprising the above IC-binding proteins, and pharmaceutical compositions comprising the IC-binding protein antagonists, for the treatment or prophylaxis of TNF-induced or FAS ligand-induced conditions, for example, such compositions can be used to enhance the TNF or FAS ligand effect or to inhibit the TNF or FAS ligand effect depending on the above noted nature of the IC-binding protein or antagonist thereof contained in the composition.

Moreover, in accordance with another aim of the present invention, there is disclosed other ways for eliminating or antagonizing endogenously formed or exogenously administered TNF or FAS-R ligand, by the use of soluble oligomeric TNF-Rs, oligomeric FAS-Rs, or oligomers being a mixture of TNF-Rs and FAS-Rs. In this respect it should be mentioned that one attempt in this direction was the isolation and recombinant production of a TNF Binding Protein called TBP-I which was shown to be able to antagonize the effects of TNF. This antagonism was determined both by measuring reduction of the cytotoxic activity of TNF, as well as by measuring interference of TNF binding to its receptors (EP 308 378). TBP-I was shown to protect cells from TNF toxicity at concentrations of a few nanograms per ml and to interfere with the binding of both TNF-α and TNF-β to cells, when applied simultaneously with these cytokines. Further examination of the mechanism by which TBP-I functions revealed that TBP-I does not interact

with the target cell, but rather blocks the function of TNF by binding TNF specifically, thus competing for TNF with the TNF receptor.

Consequently, with a different purification technique, the presence of two active components was found : one, TBP-I, and also a second TNF-binding protein which we called TBP-II (first described in EP 398327). Both proteins provide protection against the *in vitro* cytocidal effect of TNF and both bind TNF-β less effectively than TNF-α. Although in SDS PAGE analysis the two proteins, TBP-I and TBP-II, appeared to have a very similar molecular size, they could clearly be distinguished from each other by lack of immunological cross reactivity, differing N-terminal amino acid sequences and differing amino acid composition.

However, the above noted earlier soluble TNF binding proteins are monomeric and being capable of binding only one monomer of the TNF homotrimer, the natural ligand, which still permits TNF activity (i.e. incomplete neutralization) by virtue of the TNF still having two active monomers unbound by the TNF binding proteins. Further, heretofore there has been no disclosure of soluble FAS-Rs (soluble FAS-R ligand binding proteins) capable of binding to FAS-R ligand which is known to be a homotrimeric, cell-surface associated molecule.

A so-called 'death domain' of the p55-IC (Tartaglia et al., 1993) has been disclosed, but did not show, in accordance with the present invention, that the p55-IC and the 'death domain' thereof self-associates, this self-association being primarily responsible for the signaling leading to induction of cell cytotoxis. Moreover, this publication is silent on the possibility of producing the soluble, oligomeric TNF-Rs, or the soluble, oligomeric Fas-Rs, or mixed oligomeric thereof, nor does it disclose other TNF-associated effects induced by the p55-IC or portions thereof, e.g. IL-8 gene expression induction, all of the present invention. Likewise, another publication, published after the date of the present invention, disclosed the aggregation (i.e. self-association) ability of the p55-IC, but did not relate, as noted above, to the usage thereof to prepare soluble, oligomeric TNF-Rs or Fas-Rs nor to the other TNF-associated effects induced in a ligand-independent manner by the p55-IC or portions thereof according to the invention

Summary of the Invention

In accordance with the present invention, we have found novel proteins which are capable of binding to either the intracellular domain of the p55 TNF-R (the p55IC-binding proteins), of the p75 TNF-R (the p75IC-binding proteins), and of the FAS-R (the FAS-IC-binding proteins). These p55IC-, p75IC- and FAS-IC- binding proteins may act as mediators or modulators of the TNF or FAS-R ligand effect on cells by way of mediating or modulating the intracellular signaling process which usually occurs following the binding of TNF to the p55 and/or p75 TNF-R, or the binding of the FAS-R ligand at the cell surface. Further, it has been surprisingly and unexpectedly found that the p55IC and FAS-IC are capable of self association and that fragments of the p55IC and FAS-IC are similarly capable of binding to the p55 IC, particularly the so-called 'death domains (DD) within the ICs of these receptors, i.e. the p55DD and FAS-DD. Thus, p55 IC and FAS-IC and their fragments also represent proteins capable of binding to the p55IC and FAS-IC and hence may be modulators of the TNF or FAS-R ligand effect on cells.

Furthermore, the nature of the binding of one of the novel proteins of the invention, the herein designated 55.11 protein, to the intracellular domain of p55-TNF-R has been more fully elucidated (see Example 1).

Moreover, in another aspect, the present invention is based on the finding that the intracellular domain of the p55 TNF receptor (p55-IC), a region contained therein, the so-called p55-IC 'death domain', the intracellular domain of the Fas/APO1 receptor (Fas-IC), and a region contained therein, the so-called Fas-IC 'death domain' are capable of self-association. Accordingly, it is possible to construct by standard recombinant DNA techniques, a soluble, oligomeric TNF receptor being a fusion product, containing at least two extracellular domains of a TNF receptor at its one end, and at its other end at least two of the above noted self-associating intracellular domains or portions thereof, which self-associate to provide an oligomer having at least two such fusion products linked together. Such a soluble, oligomeric TNF-R is thus capable of binding two monomers of the naturally-occurring TNF homotrimer, and as such effectively neutralizes TNF activity. The neutralization of TNF activity being desirable in all of the above mentioned conditions wherein TNF is overproduced endogenously or is administered exogenously in high doses resulting in undesirable side effects. Further, the effective binding of TNF by the soluble, oligomeric receptors of the invention may also serve to allow for the binding of exogenously added TNF and its subsequent desired slow-release in conditions where TNF is administered for its beneficial effects, e.g. in tumor therapy. Likewise, it is also possible to construct by standard recombinant DNA techniques an oligomeric FAS-R being a fusion product, containing at least two extracellular domains of a FAS-R at its one end, and at its other end at least two of the above noted self-associating intracellular domains or portions thereof, which self-associate to provide an oligomer having at least two such fusion products linked together. Such an oligomeric FAS-R is thus capable of binding two monomers of the naturally occurring FAS-R ligand homotrimer, and as such effectively neutralizes FAS-R ligand activity. The neutralization of FAS-R ligand activity being desirable in all of the above mentioned conditions where excess amounts thereof are associated with undesirable side effects. In a similar fashion, and in view of recent reports indicating a possible associating between TNF and FAS-R ligand-induced effects on cells and hence also a possible association, geographically at the cell surface where they attach to their receptors, it is also possible to construct by standard recombinant DNA techniques a mixed oligomeric receptor having specificity for both TNF and FAS-R ligand. Such a mixed oligomer would be a mixture of the above noted fusion products containing at least one extracellular domain of a TNF-R and at least one extracellular domain of a FAS-R at its one end, and at its other end at least two of the above mentioned self-associating intracellular domains or portions thereof, which self-associate to provide a mixed oligomer having at least two such fusion products linked together. Such a mixed oligomer is thus capable of binding at least one monomer of TNF and one monomer of FAS-R ligand at the same time, thereby reducing or effectively neutralizing the TNF and FAS-R ligand activities at the cell surface in conditions, as noted above where excess amounts of these two cytokines are associated with undesirable cellular effects. As noted above, the FAS-R ligand is usually cell-surface-associated, and recent reports also describe

cell-surface-associated forms of TNF. Hence, these mixed TNF-R/FAS-R oligomers are especially useful for neutralization of TNF and FAS-R ligand activities at the cell surface.

Accordingly, the present invention provides a DNA sequence encoding a protein capable of binding to one or more of the intracellular domains of one or more receptors belonging to the tumor necrosis factor/nerve growth factor (TNF/NGF) superfamily of receptors.

In particular, the present invention provides a DNA sequence selected from the group consisting of:

(a) a cDNA sequence derived from the coding region of a native TNF-R intracellular domain-binding protein;

(b) DNA sequences capable of hybridization to a DNA of (a) under moderately stringent conditions and which encode a biologically active TNF-R intracellular domain-binding protein; and

(c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b) and which encode a biologically active TNF-R intracellular domain-binding protein.

The present invention also provides a DNA sequence selected from the group consisting of:

(a) a cDNA sequence derived from the coding region of a native FAS-R intracellular domain-binding protein;

(b) DNA sequences capable of hybridization to a cDNA of (a) under moderately stringent conditions and which encode a biologically active FAS-R intracellular domain-binding protein; and

(c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b) and which encode a biologically active FAS-R intracellular domain-binding protein.

In embodiments of the present invention the DNA sequences encode p55 TNF-R, p75 TNF-R and FAS-R intracellular domain-binding proteins, such as those encoding the herein designated proteins 55.1, 55.3, 55.11, 75.3, 75.16, F2, F9 and DD11.

The present invention also provides a protein or analogs or derivatives thereof encoded by any of the above sequences of the invention, said proteins, analogs and derivatives being capable of binding to one or more of the intracellular domains of one or more TNF-Rs or FAS-R. Embodiments of this aspect of the invention include the herein designated proteins 55.1, 55.3, 55.11, 75.3, 75.16, F2, F9 and DD11, their analogs and their derivatives.

Also provided by the present invention are vectors encoding the above proteins of the invention, which contain the above DNA sequences of the invention, these vectors being capable of being expressed in suitable eukaryotic or prokaryotic host cells; transformed eukaryotic or prokaryotic host cells containing such vectors; and a method for producing the proteins, analogs or derivatives of the invention by growing such transformed host cells under conditions suitable for the expression of said protein, analogs or derivatives, effecting post-translational modifications of said protein as necessary for obtention of said protein and extracting said expressed protein

analogs or derivatives from the culture medium of said transformed cells or from cell extracts of said transformed cells.

In another aspect, the present invention also provides antibodies or active derivatives or fragments thereof specific to the proteins, analogs and derivatives thereof, of the invention.

By yet another aspect of the invention, there are provided various uses of the above DNA sequences or the proteins which they encode, according to the invention, which uses include amongst others :

(i) a method for the modulation of the TNF or FAS-R ligand effect on cells carrying a TNF-R or a FAS-R, comprising treating said cells with one or more proteins, analogs or derivatives selected from the group consisting of the proteins, analogs and derivatives, according to the invention, and a protein being the p55IC, p55DD, FAS-IC or FAS-DD, analogs or derivatives thereof, all of said proteins being capable of binding to the intracellular domain and modulating the activity of said TNF-R or FAS-R, wherein said treating of the cells comprises introducing into said cells said one or more proteins, analogs or derivatives in a form suitable for intracellular administration or introducing into said cells, in the form of a suitable expression vector, the DNA sequence encoding said one or more proteins, analogs or derivatives ;

(ii) a method for modulating the TNF or FAS-R ligand effect on cells carrying a TNF-R or a FAS-R comprising treating said cells with antibodies or active derivatives or fragments thereof according to the invention;

(iii) a method for modulating the TNF or FAS-R ligand effect on cells carrying a TNF-R or FAS-R comprising treating said cells with an oligonucleotide sequence encoding an antisense sequence of at least part of the sequence according to the invention, or encoding an antisense sequence of the p55IC, p55DD, FAS-IC, or FAS-DD sequence, said oligonucleotide sequence being capable of blocking the expression of at least one of the TNF-R or FAS-R intracellular domain binding proteins;

(iv) a method for modulating the TNF or FAS-R ligand effect on cells carrying a TNF-R or FAS-R comprising :

(a) constructing a recombinant animal virus vector carrying a sequence encoding a viral surface protein that is capable of binding to a specific cell surface receptor and a sequence selected from an oligonucleotide sequence encoding an antisense sequence of at least part of the sequence according to the invention and an oligonucleotide sequence encoding an antisense sequence of the p55IC, p55DD, FAS-IC, or FAS-DD sequence, said oligonucleotide sequence being capable of blocking the expression of at least one of the TNF-R or FAS-R intracellular domain binding proteins when introduced into said cells by said virus ; and

(b) infecting said cells with said vector of (a).

(v) a method for modulating the TNF or FAS-R ligand effect on cells carrying a TNF-R or a FAS-R, comprising treating said cells with a suitable vector encoding a ribozyme having a sequence specific to a sequence selected from an mRNA sequence encoding a

protein, analog or derivative of the invention and an mRNA sequence encoding the p55IC, p55DD, FAS-IC or FAS-DD, said ribozyme sequence capable of interacting with said mRNA sequence and capable of cleaving said mRNA sequence resulting in the inhibition of the expression of the protein, analog or derivative of the invention or of the expression of the p55IC, p55DD, FAS-IC or FAS-DD.

(vi) a method for treating tumor cells or HIV-infected cells, or other diseased cells, comprising:

(a) constructing a recombinant animal virus vector carrying a sequence encoding a viral surface protein that is capable of binding to a tumor cell surface receptor or HIV-infected cell surface receptor or is capable of binding to another cell surface receptor of other diseased cells and a sequence selected from a sequence according to the invention encoding a protein, analog or derivative of the invention and a sequence encoding the p55IC, p55DD, FAS-IC, FAS-DD, or a biologically active analog or derivative thereof, said protein, analog or derivative of the invention, p55IC, p55DD, FAS-IC, FAS-DD, analog or derivative, when expressed in said tumor cell or HIV-infected cell, or other diseased cell being capable of killing said cell; and

(b) infecting said tumor cells or HIV-infected cells or other infected cells with said vector of (a).

(vii) a method for isolating and identifying proteins, factors or receptors capable of binding to the intracellular domain binding proteins according to the invention, comprising applying the procedure of affinity chromatography in which said protein according to the invention is attached to the affinity chromatography matrix, said attached protein is brought into contact with a cell extract and proteins, factors or receptors from cell extract which bound to said attached protein are then eluted, isolated analyzed;

(viii) a method for isolating and identifying proteins, capable of binding to the intracellular domain binding proteins according to the invention, comprising applying the yeast two-hybrid procedure in which a sequence encoding said intracellular domain binding protein is carried by one hybrid vector and a sequence from a cDNA or genomic DNA library is carried by the second hybrid vector, the vectors then being used to transform yeast host cells and the positive transformed cells being isolated, followed by extraction of the said second hybrid vector to obtain a sequence encoding a protein which binds to said intracellular domain binding protein; and

(ix) a method for isolating and identifying a protein capable of binding to the intracellular domains of TNF-Rs or FAS-R comprising applying the procedure of non-stringent southern hybridization followed by PCR cloning, in which a sequence or parts thereof according to the invention is used as a probe to bind sequences from a cDNA or genomic DNA library, having at least partial homology thereto, said bound sequences then amplified and cloned by the PCR procedure to yield clones encoding proteins having at least partial homology to said sequences according to the invention.

The present invention also provides a pharmaceutical composition for the modulation of the TNF- or FAS ligand- effect on cells comprising, as active ingredient, any one of the following : (i) a protein according to the invention, or the protein p55IC, p55DD, FAS-IC or FAS-DD, its biologically active fragments, analogs, derivatives or mixtures thereof; (ii) a recombinant animal virus vector encoding a viral surface protein capable of binding to a TNF-R or FAS-R - carrying cell - or tumor cell-specific receptor and a sequence encoding a protein, analog or derivative of the invention or encoding the p55IC, p55DD, FAS-IC or FAS-DD; (iii) a recombinant animal virus vector encoding a viral surface protein as in (ii) above and an oligonucleotide sequence encoding an antisense sequence of the p55IC, p55DD, FAS-IC or FAS-DD sequence; and (iv) a vector encoding a ribozyme of sequence capable of interacting with a mRNA sequence encoding a protein, analog or derivative of the invention or a mRNA sequence encoding the p55IC, p55DD, FAS-IC or FAS-DD.

A specific embodiment of the above aspects of the invention is the use of the p55-IC or DNA encoding therefor. This embodiment is based on the discovery that the p55-IC may in a ligand (TNF)-independent fashion induce other TNF-associated effects in cells. Accordingly, there is provided a method for inducing TNF-associated effects in cells or tissues comprising treating said cells with one or more proteins, analogs or derivatives thereof, said one or more proteins being selected from a protein being essentially all of the self-associating intracellular domain of the p55 TNF-R (p55-IC) or portions thereof capable of self-associating and inducing, in a ligand (TNF)-independent manner, said TNF effect in the cells, wherein said treating of the cells comprises introducing into said cells said one or more proteins, analogs or derivatives in a form suitable for intracellular introduction thereof, or introducing into said cells a DNA sequence encoding said one or more proteins, analogs or derivatives in the form of a suitable vector carrying said sequence, said vector being capable of effecting the insertion of said sequence into said cells in a way that said sequence is expressed in said cells.

Embodiments of the above method of the invention include :

(i) a method wherein said treating of cells is by transfection of said cells with a recombinant animal virus vector comprising the steps of :

(a) constructing a recombinant animal virus vector carrying a sequence encoding a viral surface protein (ligand) that is capable of binding to a specific cell surface receptor on the surface of said cells to be treated, and a second sequence encoding a protein being the p55-IC, portions thereof, analogs and derivatives of all of the foregoing, said protein when expressed in said cells being capable of self-association and induction of said one or more TNF-associated effects; and

(b) infecting said cells with the vector of (a).

(ii) a method wherein said TNF effect to be induced in said cells is the induction of IL-8 gene expression, said vector carrying a sequence encoding essentially all of said p55-IC, portions thereof, analogs and derivatives of all of the foregoing, which are capable, when expressed in the cells of self-association and signaling for the induction of said IL-8 gene expression.

(iii) a method for treating tumor cells or virally-infected cells, or for augmenting the antibacterial effect of granulocytes, wherein said viral vector carries a sequence encoding a viral ligand capable of binding a specific cell surface receptor on the surface of said tumor cells, virally-infected cells or granulocytes and a sequence encoding said p55-IC, portions thereof, analogs and derivatives thereof, which when expressed in said tumor, virally-infected or granulocyte cells induces TNF-associated effects leading to the death of these cells.

(iv) a method for treating tumor cells, wherein said p55-IC, portions thereof, analogs or derivatives thereof, when expressed in the tumor cells, induce the expression of IL-8 which leads to the killing of said tumor cells by its chemotactic activity which attracts granulocytes and other lymphocytes to the tumor cells resulting in the death of the tumor cells.

In this aspect of the invention, there is thus also provided the intracellular domain of the p55-R (p55-IC), portions, analogs and derivatives of all of the aforegoing for use in the treatment of cells by induction therein of TNF-associated effects; and the following embodiments thereof:

(i) the p55-IC, portions, analogs and derivatives for use in the treatment of cells by induction therein of IL-8 gene expression.

(ii) the p55-IC, portions, analogs and derivatives for use in the treatment of tumor cells by induction therein of IL-8 gene expression resulting in the killing of the tumor cells.

Moreover, in this aspect of the invention there is provided a pharmaceutical composition for treating cells by induction therein of TNF-associated effects, comprising, as active ingredient, p55-IC, portions thereof, analogs and derivatives of all of the aforegoing, and a pharmaceutically acceptable carrier; and the following embodiments thereof:

(i) a pharmaceutical composition for treating cells by induction therein of TNF-associated effects, comprising, as active ingredient a recombinant animal virus vector encoding p55-IC, portions thereof, analogs and derivatives of all of the aforegoing, and a protein capable of binding a cell surface protein on the cells to be treated.

(ii) a pharmaceutical composition for the treatment of tumor cells, administration of said composition leading to the induction of IL-8 expression, and subsequent killing of the tumor cells.

As yet another aspect, the present invention provides a soluble, oligomeric tumor necrosis factor receptor (TNF-R) comprising at least two self-associated fusion proteins, each fusion protein having (a) at its one end, a TNF binding domain selected from the extracellular domain of a TNF-R, analogs or derivatives thereof, said extracellular domain, analogs or derivatives thereof being incapable of deleterious self-association and being able to bind TNF; and (b) at its other end, a self-associating domain selected from (i) essentially all of the intracellular domain of the p55 TNF-R (p55-IC), extending from about amino acid residue 206 to about amino acid residue 426 of the native p55 TNF-R molecule (p55-R); (ii) the death domain of the p55-IC extending from about amino acid residue 328 to about amino acid residue 426 of the native p55-R; (iii) essentially all of the intracellular domain of the Fas/APO1 receptor (Fas-IC); (iv) the death domain of Fas-IC; and (v) analogs, fractions or derivatives of any one of (i)-(iv) being capable of self-association, wherein said at least two self-associated proteins self-associate only at said ends (b) having said ends (a) capable of binding to at least two TNF monomers, each end (a) capable

of binding one TNF monomer; and salts and functional derivatives of said soluble, oligomeric TNF-R.

Embodiments of this aspect of the invention include all of the above combinations of ends (a) with ends(b) as defined above, for example, a soluble, oligomeric TNF-R comprising as extracellular domain, the p55-R extracellular domain and as self-associating intracellular domain, the p55-IC.

Moreover, there is also provided a process for producing the soluble oligomeric TNF-R of the invention comprising :

(a) the construction of an expression vector encoding any one of said fusion proteins, the DNA sequence of each of said ends of the fusion protein being obtained from cloned DNA sequences encoding essentially all of said extracellular domain of the TNF-R, analogs or derivatives thereof; and from cloned DNA sequences encoding essentially all of said p55-IC, p55-IC death domain, Fas-IC, Fas-IC death domain, analogs or derivatives of all of the aforegoing, said ends being ligated together to form a fusion protein sequence, and said fusion protein sequence being inserted into said vector under the control of transcriptional and translational regulatory sequences;

(b) introduction of the vector of (a) into a suitable host cell in which said fusion protein is expressed; and

(c) purification of the fusion protein expressed in said host cells, said fusion protein self-associating prior to, during, or following the purification process to yield a soluble, oligomeric TNF-R.

Furthermore, there is also provided a vector encoding the above fusion proteins, useful in the above method of the invention; host cells containing the vector; as well as a pharmaceutical composition comprising the soluble, oligomeric TNF-R, salts or functional derivatives thereof and mixtures of any of the aforegoing according to the invention, as active ingredient, together with a pharmaceutically acceptable carrier. Similarly, the soluble, oligomeric TNF-R, salts, functional derivatives thereof and mixtures of any of the aforegoing, according to the invention, are provided for use in antagonizing the deleterious effect of TNF in mammals, especially in the treatment of conditions wherein an excess of TNF is formed endogenously or is exogenously administered; or alternatively, for use in maintaining prolonged beneficial effects of TNF in mammals when used with TNF exogenously administered.

Along the lines set forth concerning the above aspect of the invention, it has also been discovered that it is possible to construct a soluble, oligomeric Fas/APO1 receptor (Fas-R) which is useful for antagonizing the deleterious effects of the Fas ligand. Accordingly, in a further aspect, the present invention provides a soluble, oligomeric Fas/APO1 receptor (Fas-R) comprising at least two self-associated fusion proteins, each fusion protein having (a) at its one end, a Fas ligand binding domain selected from the extracellular domain of a Fas-R, analogs or derivatives thereof being incapable of self-associating and being able to bind Fas ligand; and (b) at its other end, a self-associating domain selected from (i) essentially all of the intracellular domain of the p55 TNF-R (p55-IC), extending from about amino acid residue 206 to about amino acid

residue 426 of the native p55 TNF-R molecule (p55-R); (ii) the death domain of the p55-IC extending from about amino acid residue 328 to about amino acid residue 426 of the native p55-R; (iii) essentially all of the intracellular domain of the Fas/APO1 receptor (Fas-IC); (iv) the death domain of Fas-IC; and (v) analogs or derivatives of any one of (i)-(iv) being capable of self-association, wherein said at least two self-associated proteins only self-associate at said ends (b) having said ends (a) capable of binding to at least two Fas ligand monomers, each end (a) capable of binding one Fas ligand monomer; and salts and functional derivatives of said soluble, oligomeric Fas-R.

In accordance with this aspect of the invention, there is also provided a process for the production of the soluble, oligomeric Fas-R comprising :

(a) the construction of an expression vector encoding any one of said fusion proteins, the DNA sequence of each of said ends of the fusion protein being obtained from cloned DNA sequences encoding essentially all of said extracellular domain of the Fas-R, analogs or derivatives thereof , and from cloned DNA sequences encoding essentially all of said p55-IC, p55-IC death domain, Fas-IC, Fas-IC death domain, analogs or derivatives thereof of all the aforegoing, said ends being ligated together to form a fusion protein sequence, and said fusion protein sequence being inserted into said vector under the control of transcriptional and translational regulatory sequences;

(b) introduction of the vector of (a) into a suitable host cell in which said fusion protein is expressed; and

(c) purification of the fusion protein expressed in the host cells, said fusion protein self-associating prior to, during, or following the purification process to yield a soluble, oligomeric Fas-R.

Moreover, also provided are an expression vector containing the fusion protein sequence encoding the soluble oligomeric Fas-R, useful in the above process; host cells containing the vector; and pharmaceutical compositions comprising the soluble, oligomeric Fas-R, salts or functional derivatives thereof or mixtures of any of the aforegoing as active ingredient together with a pharmaceutically acceptable carrier. Similarly, there is provided a soluble, oligomeric Fas-R, salts or functional derivatives thereof or mixtures of any of the aforegoing, for use in antagonizing the deleterious effect of Fas ligand in mammals, especially in the treatment of conditions wherein an excess of the Fas ligand is formed endogenously or is exogenously administered.

In a similar fashion to that noted above concerning the oligomeric TNF-Rs and oligomeric FAS-Rs, it is also possible to prepare mixed oligomers having binding specificity for both TNF and FAS-R ligand. Thus, the present invention also provides a mixed oligomeric TNF-R/FAS-R comprising at least two self-associated fusion proteins, one of which fusion proteins is selected from any one of the above mentioned TNF-specific fusion proteins, and the other fusion protein is selected from any one of the above mentioned FAS-R ligand-specific fusion proteins, to provide a mixed oligomer having at least one TNF-R extracellular domain and at least one FAS-R extracellular domain associated by virtue of the self-association between the intracellular domains

or portions thereof fused to each of these extracellular domains. These mixed oligomeric receptors are prepared by preparing, as noted above, the oligomeric TNF-Rs and the oligomeric FAS-Rs and then mixing these together and subsequently selecting, by standard procedures, those oligomers having binding specificity for both FAS-R ligand and TNF. Another way for preparing the mixed oligomeric receptors is by co-transfecting suitable host cells with vectors, as noted above, encoding any of the TNF-specific fusion proteins (soluble TNF-Rs) and encoding any of the FAS-R ligand-specific fusion proteins (soluble FAS-Rs), purifying the expressed fusion proteins which self-associate prior to, during, or following the purification to yield oligomeric receptors, and then selecting by standard procedures, those oligomeric receptors which are capable of binding to both TNF and FAS-R ligand.

Likewise, there is also provided pharmaceutical compositions comprising the mixed oligomeric receptors, salts or functional derivatives thereof or mixtures of any of the aforegoing as active ingredient together with a pharmaceutically acceptable carrier. In addition, there is provided the mixed oligomeric receptors, salts or functional derivatives thereof or mixtures of any of the aforegoing, for use in antagonizing the deleterious effects of both TNF and FAS-R ligand in mammals, especially in the treatment of conditions wherein an excess of TNF and FAS-R ligand is formed endogenously or is exogenously administered; or alternatively, for use in maintaining prolonged (slow-release) beneficial effects of TNF and/or FAS-R ligand in mammals when used with TNF and/or FAS-R ligand (in soluble form) exogenously administered.

Other aspects and embodiments of the present invention are also provided as arising from the following detailed description of the invention.

It should be noted that, where used throughout, the following terms : "Modulation of the TNF-effect on cells" and "Modulation of the FAS-ligand effect on cells" are understood to encompass *in vitro* as well as *in vivo* treatment.

Brief Description of the Drawings

Figs 1a-c depict schematically the partial and preliminary nucleotide sequence of cDNA clones encoding the p55IC and p75IC-binding proteins, wherein Fig. 1(a) is the sequence of clone 55.11 encoding the p55IC-binding protein 55.11; Fig. 1(b) is the partial and preliminary sequence of clone 75.3 encoding the p75IC-binding protein 75.3; and Fig. 1(c) is the partial and preliminary sequence of clone 75.16 encoding the p75IC-binding protein p75.16; all as described in Example 1; and Fig. 1(d) depicts the deduced amino acid sequence of protein 55.11, deduced from the nucleotide sequence of Fig. 1(a), as also described in Example 1.

Fig. 2 is a reproduction of a Northern blot which shows the 55.11-specific mRNAs present in a number of tested cell lines, as described in Example 1.

Figs. 3A and B are reproductions of autoradiograms depicting the *in vitro* binding of the protein encoded for by the 55.11 cDNA to GST fusion proteins containing portions of p55-IC, wherein in Fig. 3A there is depicted the binding of the full-length 55.11 protein (55.11 full) to the various GST fusion proteins; and in Fig. 3B there is depicted the binding of a

portion of 55.11 fused to the FLAG octapeptide to the various GST fusion proteins, all as described in Example 1.

Fig. 4 shows schematically a comparison of the deduced amino acid sequence of human 55.11 to related protein sequences derived from lower organisms, as described in Example 1.

Fig. 5 is a reproduction of a Western blot stained with anti-MBP polyclonal antiserum, showing the self association of the p55IC, the Western blot derived from an SDS-PAGE gel on which were electrophoresed the interacting bacterially-produced chimeric proteins p55IC-MBP and p55IC-GST (lanes 1-4) or the control interaction between the chimeric protein p55IC-MBP and GST alone (lanes 5-8), the interactions between the chimeric proteins (and control) being carried out on glutathion-agarose beads prior to SDS-PAGE, as described in Example 2.

Fig. 6 is a reproduction of phase contrast micrographs showing the cytotoxic effect of the full-length p55IC in HTta1 cells transfected with an expression vector encoding this p55IC (right panel); and the inhibition of this cytotoxic effect when expression of the vector is blocked by treating the cells with tetracycline (left panel), as described in Example 2.

Fig. 7 depicts the ligand-independent triggering of the cytocidal effect in HeLa cells transfected with the full-length p55-R, its intracellular domain, or parts of the intracellular domain including the 'death domain' where :

(i) at the extreme left hand side of Fig. 7 there is depicted schematically the various DNA molecules encoding the full-length p55-R, its intracellular domain and the portions of the intracellular domain which were inserted into the vector with which the HeLa cells were transfected.

(ii) the left and middle bar graphs show the TNF receptor expression in the HeLa cells of each of the types of receptor shown at the extreme left of Fig. 7, the left bar representing the amounts of receptor in ng/cell sample and the middle bar graph representing the amounts of receptor expressed in terms of radioiodinated TNF bound to the transfected cells; and

(iii) the right bar graph showing the viability of the HeLa cells expressing the various kinds of the receptor;

and wherein in all of the bar graphs the open bars represent cells transfected in the presence of tetracycline and the closed bars represent cells transfected in the absence of tetracycline; all of the above being described herein in Example 2.

Fig. 8 depicts the ligand-independent induction of IL-8 gene expression in HeLa cells transfected with the full-length p55-R or its intracellular domain (p55IC), wherein in panel A there is shown a reproduction of a Northern blot representing the Northern analysis of RNA extracted from HeLa cells treated or untreated with TNF (two left hand lanes marked 'control' and 'TNF'), and of RNA extracted from HeLa cells transfected with vectors encoding the p55-R, p55-IC or the control protein, luciferase (the remaining lanes marked 'p55-IC', 'p55-R' and Luc, respectively), the cells having been transfected in the presence (+) or absence (-) of tetracycline in each case (hence two lanes per transfection); and

wherein in panel B there is shown the methylene blue staining of 18S rRNA in each of the HeLa cell sample shown in panel A; all of the above being described in Example 2.

Fig. 9 (A and B) depicts graphically the ligand independent triggering of a cytocidal effect in HeLa cells transfected with p55R or parts thereof, or with FAS-IC, wherein in Fig. 9A there is depicted the results with respect to the p55R or parts thereof and in Fig. 9B there is depicted the results with respect to the FAS-IC. In the left hand panels of both Fig. 9A and B there is depicted schematically the portion of the p55R or FAS-IC used in the transfections while the right hand panels depict graphically the experimental results, all as described in Example 2.

Fig. 10 depicts schematically the partial and preliminary nucleotide sequence of a cDNA clone, called 'F2', which encodes a protein capable of binding to the p55IC and FAS-IC, as described in Example 3.

Fig. 11 depicts schematically the partial and preliminary nucleotide sequence of a cDNA clone, called F9, which encodes a protein capable of binding to the p55IC and FAS-IC, as described in Example 3.

Fig. 12 depicts schematically the partial and preliminary nucleotide sequence of a cDNA clone, called DD11, which encodes a protein capable of binding to the p55IC, especially the p55DD, and FAS-IC, as described in Example 3.

Detailed Description of the Invention

The present invention relates, in one aspect, to novel proteins which are capable of binding to the intracellular domain of receptors belonging to the TNF/NGF superfamily, such as TNF-Rs and FAS-R and hence are considered as mediators or modulators of this superfamily of receptors, e.g. of the TNF-Rs and FAS-R, having a role in, for example, the signaling process that is initiated by the binding of TNF to the TNF-R and FAS ligand to FAS-R. Examples of these proteins are those which bind to the intracellular domain of the p55 TNF-R (p55IC), such as the proteins designated herein as 55.1, 55.3 and 55.11 (Example 1) as well as those encoded by cDNA clones F2, F9, and DD11 (Example 3); those which bind to the intracellular domain of the p75 TNF-R (p75IC), such as the proteins designated herein as 75.3 and 75.16 (Example 1); and those which bind to the intracellular domain of FAS-R (FAS-IC), such as the proteins encoded by cDNA clones F2, F9 and DD11 (Example 3). Proteins 55.1 and 55.3 have been found to represent portions or fragments of the intracellular domain of the p55 TNF-R (p55IC); other proteins, 55.11, 75.3 and 75.16, represent proteins not described at all prior to the present invention (75.3, 75.16) or those that have been described (55.11, see Khan et al., 1992) but whose function and other characteristics, particularly, the ability to bind to a TNF-R, were not described in any way (see Example 1, below). The new proteins encoded by cDNA clones F2, F9 and DD11 also represent proteins previously not described at all, i.e. their sequence is not in the 'GENEBANK' or 'PROTEIN BANK' data banks of DNA or amino acid sequences.

Thus, the present invention concerns the DNA sequences encoding these proteins and the proteins encoded by these sequences.

Moreover, the present invention also concerns the DNA sequences encoding biologically active analogs and derivatives of these proteins, and the analogs and derivatives encoded thereby. The preparation of such analogs and derivatives is by standard procedure (see for example, Sambrook et al., 1989) in which in the DNA sequences encoding these proteins, one or more codons may be deleted, added or substituted by another, to yield analogs having at least a one amino acid residue change with respect to the native protein. Acceptable analogs are those which retain at least the capability of binding to the intracellular domain of the TNF/NGF receptor superfamily, such as FAS-R or TNF-R, e.g. the p55IC, p75IC or FAS-IC, or which can mediate any other binding or enzymatic activity, e.g. analogs which bind the p55, p75IC or FAS-IC but which do not signal, i.e. do not bind to a further downstream receptor, protein or other factor, or do not catalyze a signal-dependent reaction. In such a way analogs can be produced which have a so-called dominant-negative effect, namely, an analog which is defective either in binding to the, for example, p55IC, p75IC or FAS-IC, or in subsequent signaling following such binding. Such analogs can be used, for example, to inhibit the TNF- or FAS-ligand- effect by competing with the natural IC-binding proteins. Likewise, so-called dominant-positive analogs may be produced which would serve to enhance, for example, the TNF or FAS ligand effect. These would have the same or better IC-binding properties and the same or better signaling properties of the natural IC-binding proteins. Similarly, derivatives may be prepared by standard modifications of the side groups of one or more amino acid residues of the proteins, or by conjugation of the proteins to another molecule e.g. an antibody, enzyme, receptor, etc., as are well known in the art.

The new TNF-R and FAS-R intracellular domain - binding proteins, e.g. the proteins 55.1, 55.3, 55.11, 75.3, 75.16 as well as the proteins encoded by cDNA clones F2, F9 and DD11 (hereinafter, F2, F9 and DD11) have a number of possible uses, for example:

(i) They may be used to mimic or enhance the function of TNF or FAS-R ligand, in situations where an enhanced TNF or FAS-R ligand effect is desired such as in anti-tumor, anti-inflammatory or anti-HIV applications where the TNF-or FAS-R ligand- induced cytotoxicity is desired. In this case the proteins, e.g. those binding to the p55IC such as 5.1, 55.3, as well as F2, F9 and DD11, and the free p55IC itself (see below and Example 2), as well as the 'death domain' of the p55IC (p55DD), which enhance the TNF effect; or proteins F2, F9 and DD11 as well as FAS-IC and FAS-DD which enhance the FAS-R ligand effect, i.e. cytotoxic effect, may be introduced to the cells by standard procedures known per se. For example, as the proteins are intracellular and it is desired that they be introduced only into the cells where the TNF or FAS-R ligand effect is wanted, a system for specific introduction of these proteins into the cells is necessary. One way of doing this is by creating a recombinant animal virus e.g. one derived from Vaccinia, to the DNA of which the following two genes will be introduced: the gene encoding a ligand that binds to cell surface proteins specifically expressed by the cells e.g. ones such as the AIDs (HIV) virus gp120 protein which binds specifically to some cells (CD4 lymphocytes and related leukemias) or any other ligand that binds specifically to cells carrying a TNF-R or FAS-R, such that the recombinant virus vector will be capable of binding such TNF-R- or FAS-R-

carrying cells; and the gene encoding the new intracellular domain-binding protein or the p55IC, p55DD, FAS-IC or FAS-DD protein. Thus, expression of the cell-surface-binding protein on the surface of the virus will target the virus specifically to the tumor cell or other TNF-R- or FAS-R- carrying cell, following which the intracellular domain-binding protein encoding sequence or p55IC, p55DD, FAS-IC or FAS-DD encoding sequence will be introduced into the cells via the virus, and once expressed in the cells will result in enhancement of the TNF or FAS-R ligand effect leading to the death of the tumor cells or other TNF-R- or FAS-R- carrying cells it is desired to kill. Construction of such recombinant animal virus is by standard procedures (see for example, Sambrook et al., 1989). Another possibility is to introduce the sequences of the new proteins or the p55IC, p55DD, FAS-IC or FAS-DD in the form of oligonucleotides which can be absorbed by the cells and expressed therein.

(ii) They may be used to inhibit the TNF or FAS-R ligand effect, e.g. in cases such as tissue damage in septic shock, graft-vs.-host rejection, or acute hepatitis, in which case it is desired to block the TNF-induced TNF-R or FAS-R ligand induced FAS-R intracellular signaling. In this situation it is possible, for example, to introduce into the cells, by standard procedures, oligonucleotides having the anti-sense coding sequence for these new proteins, or the anti-sense coding sequence for p55IC, p55DD, FAS-IC or FAS-DD, which would effectively block the translation of mRNAs encoding these proteins and thereby block their expression and lead to the inhibition of the TNF- or FAS-R ligand-effect.

Such oligonucleotides may be introduced into the cells using the above recombinant virus approach, the second sequence carried by the virus being the oligonucleotide sequence. Another possibility is to use antibodies specific for these proteins to inhibit their intracellular signaling activity. It is possible that these new proteins have an extracellular domain as well as an intracellular one, the latter which binds to the TNF-R or FAS-R binding domain, and thus antibodies generated to their extracellular domains can be used to block their TNF- or FAS-R ligand- related functions.

Yet another way of inhibiting the TNF or FAS-R ligand effect is by the recently developed ribozyme approach. Ribozymes are catalytic RNA molecules that specifically cleave RNAs. Ribozymes may be engineered to cleave target RNAs of choice, e.g. the mRNAs encoding the new proteins of the invention or the mRNA encoding the p55IC, p55DD, FAS-IC or FAS-DD. Such ribozymes would have a sequence specific for the mRNA of choice and would be capable of interacting therewith (complementary binding) followed by cleavage of the mRNA, resulting in a decrease (or complete loss) in the expression of the protein it is desired to inhibit, the level of decreased expression being dependent upon the level of ribozyme expression in the target cell. To introduce ribozymes into the cells of choice (e.g. those carrying TNF-Rs or FAS-R) any suitable vector may be used, e.g. plasmid, animal virus (retrovirus) vectors, that are usually used for this purpose (see also (i) above, where the virus has, as second sequence, a cDNA

encoding the ribozyme sequence of choice). Moreover, ribozymes can be constructed which have multiple targets (multi-target ribozymes) that can be used, for example, to inhibit the expression of one or more of the proteins of the invention and/or the p55IC, p55DD, FAS-IC or FAS-DD as well (For reviews, methods etc. concerning ribozymes see Chen et al., 1992; Zhao and Pick, 1993; Shore et al., 1993. Joseph and Burke, 1993; Shimayama et al., 1993; Cantor et al., 1993; Barinaga, 1993; Crisell et al., 1993 and Koizumi et al., 1993).

(iii) They may be used to isolate, identify and clone other proteins which are capable of binding to them, e.g. other proteins involved in the intracellular signaling process that are downstream of the TNF-R or FAS-R intracellular domain In this situation, these options, namely, the DNA sequences encoding them may be used in the yeast two-hybrid system (see Example 1, below) in which the sequence of these proteins will be used as "baits" to isolate, clone and identify from cDNA or genomic DNA libraries other sequences ("preys") encoding proteins which can bind to these new TNF-R or FAS-R intracellular domain-binding proteins. In the same way, it may also be determined whether the specific proteins of the present invention, namely, those which bind to the p55IC, p75IC, or FAS-IC, can bind to other receptors of the TNF/NGF superfamily of receptors. For example, it has recently been reported (Schwalb et al., 1993; Baens et al., 1993; Crowe et al., 1994) that there exist other TNF-Rs besides the p55 and p75 TNF-Rs. Accordingly, using the yeast two-hybrid system it may be specifically tested whether the proteins of the present invention are capable of specifically binding to these other TNF-Rs or other receptors of the TNF/NGF superfamily. Moreover, this approach may also be taken to determine whether the proteins of the present invention are capable of binding to other known receptors in whose activity they may have a functional role.

(iv) The new proteins may also be used to isolate, identify and clone other proteins of the same class i.e. those binding to TNF-R or FAS-R intracellular domains or to functionally related receptors, and involved in the intracellular signaling process. In this application the above noted yeast two-hybrid system may be used, or there may be used a recently developed (Wilks et al., 1989) system employing non-stringent southern hybridization followed by PCR cloning. In the Wilks et al. publication, there is described the identification and cloning of two putative protein-tyrosine kinases by application of non-stringent southern hybridization followed by cloning by PCR based on the known sequence of the kinase motif, a conceived kinase sequence. This approach may be used, in accordance with the present invention using the sequences of the new proteins to identify and clone those of related TNF-R, FAS-R or related receptor (TNF/NGF superfamily receptors) intracellular domain-binding proteins.

(v) Yet another approach to utilizing the new proteins of the invention is to use them in methods of affinity chromatography to isolate and identify other proteins or factors to which they are capable of binding, e.g. other receptors related to TNF-Rs (TNF/NGF receptor superfamily) or other proteins or factors involved in the intracellular signaling

process. In this application, the proteins of the present invention, may be individually attached to affinity chromatography matrices and then brought into contact with cell extracts or isolated proteins or factors suspected of being involved in the intracellular signaling process. Following the affinity chromatography procedure, the other proteins or factors which bind to the new proteins of the invention, can be eluted, isolated and characterized.

(vi) As noted above, the new proteins of the invention may also be used as immunogens (antigens) to produce specific antibodies thereto. These antibodies may also be used for the purposes of purification of the new proteins either from cell extracts or from transformed cell lines producing them. Further, these antibodies may be used for diagnostic purposes for identifying disorders related to abnormal functioning of the TNF or FAS-R ligand system, e.g. overactive or underactive TNF- or FAS-R ligand- induced cellular effects. Thus, should such disorders be related to a malfunctioning intracellular signaling system involving the new proteins, such antibodies would serve as an important diagnostic tool.

It should also be noted that the isolation, identification and characterization of the new proteins of the invention may be performed using any of the well known standard screening procedures. For example, one of these screening procedures, the yeast two-hybrid procedure as is set forth in the following examples (Examples 1 and 3), was used to identify the new proteins of the invention. Likewise as noted above and below, other procedures may be employed such as affinity chromatography, DNA hybridization procedures, etc. as are well known in the art, to isolate, identify and characterize the new proteins of the invention or to isolate, identify and characterize additional proteins, factors, receptors, etc. which are capable of binding to the new proteins of the invention or to the receptors belonging to the TNF/NGF family of receptors.

As regards the antibodies mentioned herein throughout, the term "antibody" is meant to include polyclonal antibodies, monoclonal antibodies (mAbs), chimeric antibodies, anti-idiotypic (anti-Id) antibodies to antibodies that can be labeled in soluble or bound form, as well as fragments thereof provided by any known technique, such as, but not limited to enzymatic cleavage, peptide synthesis or recombinant techniques.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen. A monoclonal antibody contains a substantially homogeneous population of antibodies specific to antigens, which populations contains substantially similar epitope binding sites. MAbs may be obtained by methods known to those skilled in the art. See, for example Kohler and Milstein, Nature, 256:495-497 (1975); U.S. Patent No. 4,376,110; Ausubel et al., eds., Harlow and Lane ANTIBODIES : A LABORATORY MANUAL, Cold Spring Harbor Laboratory (1988); and Colligan et al., eds., Current Protocols in Immunology, Greene publishing Assoc. and Wiley Interscience N.Y., (1992, 1993), the contents of which references are incorporated entirely herein by reference. Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, GILD and any subclass thereof. A

hybridoma producing a mAb of the present invention may be cultivated *in vitro*, *in situ* or *in vivo*. Production of high titers of mAbs *in vivo* or *in situ* makes this the presently preferred method of production.

Chimeric antibodies are molecules different portions of which are derived from different animal species, such as those having the variable region derived from a murine mAb and a human immunoglobulin constant region. Chimeric antibodies are primarily used to reduce immunogenicity in application and to increase yields in production. for example, where murine mAbs have higher yields from hybridomas but higher immunogenicity in humans, such that human/murine chimeric mAbs are used. Chimeric antibodies and methods for their production are known in the art (Cabilly et al., *Proc. Natl. Acad. Sci. USA* 81:3273-3277 (1984); Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984); Boulianne et al., *Nature* 312:643-646 (1984); Cabilly et al., European Patent Application 125023 (published November 14, 1984); Neuberger et al., *Nature* 314:268-270 (1985); Taniguchi et al., European Patent Application 171496 (published February 19, 1985); Morrison et al., European Patent Application 173494 (published March 5, 1986); Neuberger et al., PCT Application WO 8601533, (published March 13, 1986); Kudo et al., European Patent Application 184187 (published June 11, 1986); Sahagan et al., *J. Immunol.* 137:1066-1074 (1986); Robinson et al., International Patent Application No. WO8702671 (published May 7, 1987); Liu et al., *Proc. Natl. Acad. Sci USA* 84:3439-3443 (1987); Sun et al., *Proc. Natl. Acad. Sci USA* 84:214-218 (1987); Better et al., *Science* 240:1041-1043 (1988); and Harlow and Lane, ANTIBODIES :A LABORATORY MANUAL, supra. These references are entirely incorporated herein by reference.

An anti-idiotypic (anti-Id) antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding site of an antibody. An Id antibody can be prepared by immunizing an animal of the same species and genetic type (e.g. mouse strain) as the source of the mAb with the mAb to which an anti-Id is being prepared. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants (the anti-Id antibody). See, for example, U.S. Patent No. 4,699,880, which is herein entirely incorporated by reference.

The anti-Id antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id may be epitopically identical to the original mAb which induced the anti-Id. Thus, by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Accordingly, mAbs generated against the IC-binding proteins, analogs or derivatives thereof, of the present invention or the p55IC, p55DD, FAS-IC, FAS-DD, analogs or derivatives thereof may be used to induce anti-Id antibodies in suitable animals, such as BALB/c mice. Spleen cells from such immunized mice are used to produce anti-Id hybridomas secreting anti-Id mAbs. Further, the anti-Id mAbs can be coupled to a carrier such as keyhole limpet hemocyanin (KLH) and used to immunize additional BALB/c mice. Sera from these mice will contain anti-anti-Id antibodies that have the binding properties of the original mAb specific for an epitope of the

above IC-binding proteins, analogs or derivatives or p55IC, p55DD, FAS-IC or FAS-DD, analogs or derivatives.

The anti-Id mAbs thus have their own idiotypic epitopes, or "idiotopes" structurally similar to the epitope being evaluated, such as GRB protein-α.

The term "antibody" is also meant to include both intact molecules as well as fragments thereof, such as, for example, Fab and $F(ab')_2$, which are capable of binding antigen. Fab and $F(ab')_2$ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody (Wahl et al., *J. Nucl. Med.* 24:316-325 (1983)).

It will be appreciated that Fab and $F(ab')_2$ and other fragments of the antibodies useful in the present invention may be used for the detection and quantitation of the IC-binding proteins or p55IC, p55DD, FAS-IC or FAS-DD according to the methods disclosed herein for intact antibody molecules. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce $F(ab')_2$ fragments).

An antibody is said to be "capable of binding" a molecule if it is capable of specifically reacting with the molecule to thereby bind the molecule to the antibody. The term "epitope" is meant to refer to that portion of any molecule capable of being bound by an antibody which can also be recognized by that antibody. Epitopes or "antigenic determinants" usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics.

An "antigen" is a molecule or a portion of a molecule capable of being bound by an antibody which is additionally capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. An antigen may have one or more than one epitope. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens.

The antibodies, including fragments of antibodies, useful in the present invention may be used to quantitatively or qualitatively detect the IC-binding proteins or p55IC, p55DD, FAS-IC, FAS-DD in a sample or to detect presence of cells which express the IC-binding proteins of the present invention or the p55IC, p55DD, FAS-IC, FAS-DD proteins. This can be accomplished by immunofluorescence techniques employing a fluorescently labeled antibody (see below) coupled with light microscopic, flow cytometric, or fluorometric detection.

The antibodies (or fragments thereof) useful in the present invention may be employed histologically, as in immunofluorescence or immunoelectron microscopy, for *in situ* detection of IC-binding proteins of the present invention or the p55IC, p55DD, FAS-IC, FAS-DD. *In situ* detection may be accomplished by removing a histological specimen from a patient, and providing the labeled antibody of the present invention to such a specimen. The antibody (or fragment) is preferably provided by applying or by overlaying the labeled antibody (or fragment) to a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the IC-binding proteins or the p55IC, p55DD, FAS-IC, FAS-DD, but also its

distribution on the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

Such assays for IC-binding proteins of the present invention or the p55IC, p55DD, FAS-IC, FAS-DD, typically comprises incubating a biological sample, such as a biological fluid, a tissue extract, freshly harvested cells such as lymphocytes or leukocytes, or cells which have been incubated in tissue culture, in the presence of a detectably labeled antibody capably of identifying the IC-binding proteins or the p55IC, p55DD, FAS-IC, FAS-DD, and detecting the antibody by any of a number of techniques well known in the art.

The biological sample may be treated with a solid phase support or carrier such as nitrocellulose, or other solid support or carrier which is capable of immobilizing cells, cell particles or soluble proteins. The support or carrier may then be washed with suitable buffers followed by treatment with a detectably labeled antibody in accordance with the present invention, as noted above. The solid phase support or carrier may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on said solid support or carrier may then be detected by conventional means.

By "solid phase support", "solid phase carrier", "solid support", "solid carrier", "support" or "carrier" is intended any support or carrier capable of binding antigen or antibodies. Well-known supports or carriers, include glass, polystyrene, polypropylene, polyethylene, dextran, nylon amylases, natural and modified celluloses, polyacrylamides, gabbros and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support or carrier configuration may be spherical, as in a bead, cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports or carriers include polystyrene beads. Those skilled in the art will know may other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of antibody, of the invention as noted above, may be determined according to well known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

Other such steps as washing, stirring, shaking, filtering and the like may be added to the assays as is customary or necessary for the particular situation.

One of the ways in which an antibody in accordance with the present invention can be detectably labeled is by linking the same to an enzyme and use in an enzyme immunoassay (EIA). This enzyme, in turn, when later exposed to an appropriate substrate, will react with the substrate in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or by visual means. Enzymes which can be used detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-

5-steroid isomeras, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection may be accomplished using any of a variety of other immunoassays. For example, by radioactivity labeling the antibodies or antibody fragments, it is possible to detect R-PTPase through the use of a radioimmunoassay (RIA). A good description of RIA may be found in Laboratory Techniques and Biochemistry in Molecular Biology, by Work, T.S. et al., North Holland Publishing Company, NY (1978) with particular reference to the chapter entitled "An Introduction to Radioimmune Assay and Related Techniques" by Chard, T., incorporated by reference herein. The radioactive isotope can be detected by such means as the use of a γ counter or a scintillation counter or by autoradiography.

It is also possible to label an antibody in accordance with the present invention with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wavelength, its presence can be then detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrine, pycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}E, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriamine pentaacetic acid (ETPA).

The antibody can also be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

An antibody molecule of the present invention may be adapted for utilization in an immunometric assay, also known as a "two-site" or "sandwich" assay. In a typical immunometric assay, a quantity of unlabeled antibody (or fragment of antibody) is bound to a solid support or carrier and a quantity of detectably labeled soluble antibody is added to permit detection and/or quantitation of the ternary complex formed between solid-phase antibody, antigen, and labeled antibody.

Typical, and preferred, immunometric assays include "forward" assays in which the antibody bound to the solid phase is first contacted with the sample being tested to extract the antigen from the sample by formation of a binary solid phase antibody-antigen complex. After a suitable incubation period, the solid support or carrier is washed to remove the residue of the fluid sample, including unreacted antigen, if any, and the contacted with the solution containing an unknown quantity of labeled antibody (which functions as a "reporter molecule"). After a second incubation period to permit the labeled antibody to complex with the antigen bound to the solid support or carrier through the unlabeled antibody, the solid support or carrier is washed a second time to remove the unreacted labeled antibody.

In another type of "sandwich" assay, which may also be useful with the antigens of the present invention, the so-called "simultaneous" and "reverse" assays are used. A simultaneous assay involves a single incubation step as the antibody bound to the solid support or carrier and labeled antibody are both added to the sample being tested at the same time. After the incubation is completed, the solid support or carrier is washed to remove the residue of fluid sample and uncomplexed labeled antibody. The presence of labeled antibody associated with the solid support or carrier is then determined as it would be in a conventional "forward" sandwich assay.

In the "reverse" assay, stepwise addition first of a solution of labeled antibody to the fluid sample followed by the addition of unlabeled antibody bound to a solid support or carrier after a suitable incubation period is utilized. After a second incubation, the solid phase is washed in conventional fashion to free it of the residue of the sample being tested and the solution of unreacted labeled antibody. The determination of labeled antibody associated with a solid support or carrier is then determined as in the "simultaneous" and "forward" assays.

The new proteins of the invention once isolated, identified and characterized by any of the standard screening procedures, for example, the yeast two-hybrid method, affinity chromatography, and any other well known method known in the art, may then be produced by any standard recombinant DNA procedure (see for example, Sambrook, et al., 1989) in which suitable eukaryotic or prokaryotic host cells are transformed by appropriate eukaryotic or prokaryotic vectors containing the sequences encoding for the proteins. Accordingly, the present invention also concerns such expression vectors and transformed hosts for the production of the proteins of the invention. As mentioned above, these proteins also include their biologically active analogs and derivatives, and thus the vectors encoding them also include vectors encoding analogs of these proteins, and the transformed hosts include those producing such analogs. The derivatives of these proteins are the derivatives produced by standard modification of the proteins or their analogs, produced by the transformed hosts.

In another aspect, the invention relates to the use of the free intracellular domain of the p55 TNR-R (p55IC) or FAS-R (FAS-IC) or their so-called 'death domains' (p55DD or FAS-DD, respectively) as an agent for enhancing the TNF or FAS-R ligand effect on cells, on its own (see Example 2). Where it is desired to introduce a TNF- or FAS-R-ligand- induced cytotoxic effect in cells, e.g. cancer cells or HIV-infected cells, the p55IC, p55DD, FAS-IC or FAS-DD can be introduced into such cells using the above noted (see (i) above) recombinant animal virus (e.g.

vaccinia) approach. Here too, the native p55IC, p55DD, FAS-IC or FAS-DD, biologically active analogs and derivatives or fragments may be used, all of which can be prepared as noted above.

Likewise, the present invention also relates to the specific blocking of the TNF-effect or FAS-R ligand-effect by blocking the activity of the p55IC, p55DD, FAS-IC or FAS-DD, e.g. anti-sense oligonucleotides may be introduced into the cells to block the expression of the p55IC, p55DD, FAS-IC or FAS-DD.

The present invention also relates to pharmaceutical compositions comprising recombinant animal virus vectors encoding the TNF-R or FAS-R intracellular domain binding proteins (including the p55IC, p55DD, FAS-IC and FAS-DD), which vector also encodes a virus surface protein capable of binding specific target cell (e.g. cancer cells) surface proteins to direct the insertion of the intracellular domain binding protein sequences into the cells.

In another aspect, the present invention also concerns, specifically, the effects of the self-associating intracellular domain of the p55 TNF receptor (p55-IC, see Example 2). An example of such effects, which is an effect normally mediated by TNF binding to its receptor and which is mimicked by the signaling activity of the self-associating p55-IC or parts thereof, is the induction of expression of the gene encoding IL-8.

IL-8 is a cytokine belonging to the subclass of chemokines having primarily chemotactic activity, and has been shown to play a major role in the chemotaxis of granulocytes and other cell types associated with a number of pathological states (see for example, Endo et al., 1994; Sekido et al., 1993; Harada et al., 1993; Ferrick et al., 1991).

TNF has a beneficial activity, and is used as such, in treatments to destroy tumor cells and virus infected cells or to augment antibacterial activities of granulocytes. However, as noted above, TNF also has undesirable activities in which case it is desired to block its activity, including those situations where large doses of TNF are used in cancer therapy, antiviral therapy or antibacterial therapy.

Accordingly, it is desirable to be able to direct TNF or a substance capable of mimicking its beneficial activity to the cells or tissues that it is specifically desired to treat.

In accordance with the present invention it has been found that the self-associating intracellular domain of the p55-R (p55-IC) can, in a ligand-independent manner, mimic a number of effects of TNF, e.g. the 'death domain' of p55-IC can induce cytotoxic effects on cells, and that the p55-IC can induce IL-8 gene expression. Thus, it is possible to utilize the p55-IC to mimic TNF function in a site-directed fashion, i.e. to introduce the p55-IC only to those cells or tissues it is desired to treat.

One example of the above approach, as mentioned above, is to specifically transfect (transform) tumor cells or malignant tissue with a DNA molecule encoding p55-IC or a portion thereof which can induce not only cytotoxic effects on such cells or tissue but also augment these effects by the co-induction of IL-8, which will result in the accumulation at the site of these cells or tissue of granulocytes and other lymphocytes, which, in turn, will serve to destroy the tumor cells or tissue. This approach obviates the need for administration of large doses of TNF with its associated deleterious side-effects.

Using conventional r combinant DNA technology, it is possible to prepare various regions of the p55-IC and to determine which region is responsible for each TNF-induced effect, e.g. we have determined that the 'death domain' is responsible for cytotoxicity (Example 2), and we have already prepared various other constructs containing portions of the p55-IC, which portions (together with part or all of the death domain) may be responsible for other TNF-effects, and which may be used in a ligand-independent manner, once self-associated for activity, to induce these effects, e.g. IL-8 induction.

It should be noted that the sequence of the p55-IC involved in the induction of other TNF-associated effects (e.g. IL-8 induction) may be different to that involved in cytotoxicity, i.e. may include none or only part of the 'death domain' and have other sequence motifs from other regions of the intracellular domain, or may be the same sequence, different features of the sequence (same sequence motif) being involved in the induction of different effects.

Accordingly, as detailed above and below, expression vectors containing these p55-IC portions, analogs or derivatives thereof may be prepared, expressed in host cells, purified and tested for their activity. In this way, a number of such p55-IC fragments having one or more TNF-associated activities may be prepared and used in a differential fashion for the treatment of any number of pathological conditions, e.g. viral infections, bacterial infections, tumors, etc. In all of these situations the specific activity can be augmented by incorporation (or co-transfection) with the p55-IC fragment responsible for IL-8 gene expression induction, permitting the desirable IL-8 chemotactic activity to enhance the destruction of the cells or tissues it is desired to destroy.

Thus, without administering systemically TNF, it is possible to induce its desirable effects by specifically introducing all or part of the p55-IC into the cells or tissues it is desired to treat.

The p55-IC may be introduced specifically into the cells or tissues it is wished to destroy by any one of the abovementioned procedures. For example, one way of doing this is by creating a recombinant animal virus e.g. one derived from Vaccinia, to whose DNA the following two genes will be introduced : the gene encoding a ligand that binds to cell surface proteins specifically expressed by the cells e.g. ones such as the AIDS virus gp120 protein which binds specifically to some cells (CD4 lymphocytes and related leukemias) or any other ligand that binds specifically to cells carrying a TNF-R, such that the recombinant virus vector will be capable of binding such TNF-R-carrying cells; and the gene encoding the p55-IC or a portion thereof. Thus, expression of the cell-surface-binding protein on the surface of the virus will target the virus specifically to the tumor cell or other TNF-R-carrying cell, following which the p55-IC, or portion thereof, encoding sequence will be introduced into the cells via the virus, and once expressed in the cells will result in enhancement of the TNF effect leading to the death of the tumor cells or other TNF-R-carrying cells it is desired to kill or induction, for example, of IL-8 which will lead to cell death. Construction of such recombinant animal virus is by standard procedures (see for example, Sambrook et al., 1989). Another possibility is to introduce the sequences of the p55-IC or parts thereof in the form of oligonucleotides which can be absorbed by the cells and expressed therein.

The present invention thus also relates specifically to pharmaceutical compositions comprising the above recombinant animal virus vectors encoding the p55-IC or portions thereof, which vector also encodes a virus surface protein capable of binding specific target cell (e.g. cancer cells) surface proteins to direct the insertion of the p55-IC, or portions thereof, sequence into the cells.

The present invention relates, in yet another aspect, to new synthetic TNF receptors which are soluble and capable of oligomerization to form dimeric, and possibly also high order multimeric, TNF receptor molecules, each monomeric part of these receptors being capable of binding to a TNF monomer. TNF occurs naturally as a homotrimer containing three, active TNF monomers, each capable of binding to a single TNF receptor molecule, while TNF receptors occur naturally as monomers each capable of binding only one of the monomers of the TNF homotrimeric molecule. Thus, when TNF binds to TNF receptors on the cell surface, it is capable of binding to three receptor molecules resulting in the clustering of the TNF receptors, which is believed to be the start of the signaling process which ultimately triggers the observed TNF effects on the cells.

While TNF has many desirable effects such as its ability to destroy, for example, tumor cells or virus-infected cells and to augment antibacterial activities of granulocytes, TNF does however, have many undesirable effects such as, for example, in many severe diseases including autoimmune disorders, rheumatoid arthritis, graft-versus-host reaction (graft rejection), septic shock, TNF has been implicated as the major cause for pathological tissue destruction. TNF may also cause excessive loss of weight (cachexia) by suppressing the activities of adipocytes. Moreover, even when administered for its desirable activities, e.g. in the treatment of various malignant or viral diseases, the dosages of TNF used are often high enough to cause within the patient a number of undesirable cytotoxic side-effects, e.g. the destruction of healthy tissue.

Accordingly, in all of the above instances where TNF action is undesirable, an effective inhibitor of TNF has been sought. Many TNF-blocking agents have been proposed, including soluble proteins capable of binding TNF and inhibiting its binding to its receptors and hence also inhibiting the cytotoxic effects of TNF (see EP 308378, EP 398327 and EP 568925). However, these TNF binding proteins, or soluble TNF receptors are monomeric, each binding only one of the TNF monomers of the TNF homotrimer. Hence, the blocking of the TNF function may not be complete, each monomeric receptor-bound TNF molecule still having two TNF monomers free to be able to bind cell-surface TNF receptors and illicit its effects on the cells.

In order to overcome the above drawbacks in blocking TNF function, there has been developed in accordance with the present invention a means for constructing, as fusion proteins, soluble oligomeric TNF receptors which are capable of binding at least two TNF monomers of the naturally occurring TNF homotrimer molecule. As a consequence, these soluble oligomeric TNF receptors bind more avidly to their TNF ligand than the previously known monomeric soluble TNF binding proteins or receptors. For example, when the soluble TNF receptor of the invention is in the form of a dimer, it is capable of binding two TNF monomers of a TNF trimer and hence causes a more complete neutralization of the TNF, this neutralization being more sustained

because of a lower dissociation rate of the dimeric soluble receptors from the TNF. Moreover, such soluble, oligomeric receptors are also larger than their monomeric counterparts and thus, pharmaceutically, they are also advantageous because of the likelihood of their having a slower clearance rate from the body.

The basis for the development of the soluble oligomeric TNF receptors of the invention, was the discovery that the intracellular domain of the p55-R TNF receptor was capable of self-association, and further, that within this intracellular domain (p55-IC) there exists a region, the so-called 'death domain', which is also capable of self-association and as such, in a ligand-independent fashion, can cause cytotoxic effects on cells (see Example 2). Utilizing this self-association property of the p55-IC and its 'death domain' it is thus possible to construct a fusion protein, using standard recombinant DNA technology, containing essentially all of the extracellular domain of a TNF receptor such as the p75-R or p55-R receptors, preferably the p55-R, and fused thereto, essentially all of the intracellular domain (p55-IC) or the death domain of the p55-IC. In this way a new fusion product is produced which has at one end the TNF binding domain i.e., the extracellular domain of the receptor, and at its other end the intracellular domain or the death domain thereof which is capable of self-association. Accordingly, such a product can oligomerize by self-association between two (and possible more) p55-IC or death domains thereof to yield oligomers (or at least dimers) having at least two TNF binding domains.

Furthermore, it has also been discovered in accordance with the present invention, that the Fas/APO1 receptor has a self-associating, intracellular domain inclusive of a self-associating 'death domain' having certain homology to the p55-IC and death domain thereof (Example 2). Accordingly, it is possible to construct the soluble, oligomeric TNF receptors of the invention by fusing the extracellular domain of the TNF receptor (as noted above) to the intracellular domain or the 'death domain' of the Fas/APO1 receptor.

In both of the above noted situations, the oligomeric TNF receptors of the invention are soluble by virtue of having only the soluble extracellular domain of the TNF receptor and the soluble intracellular domain or death domain thereof of either the p55-R TNF receptor or the Fas/APO1 receptor, i.e. they do not contain the transmembranal (insoluble) domain of either type of receptor.

The construction of the above oligomeric TNF receptors of the invention are detailed herein below in Example 4. It should however be noted that upon construction of the oligomeric TNF receptors of the invention, there may arise a situation, heretofore not reported, that the extracellular domain of the TNF receptor is capable of self-association, a situation that may not be desirable as it could interfere with the ability of the oligomeric receptor to bind to two or more TNF monomers of the TNF homotrimeric molecules or may lead to less than optimal binding of such TNF monomers. Accordingly, in such a situation, it is possible, by standard recombinant DNA procedures, to modify the extracellular domain of the TNF receptor by, for example, deleting or substituting one or more amino acid residues contained within the self-associating region to prevent such self-association. Such modifications of the extracellular domain of the TNF receptor are thus also part of the present invention and are designated herein as analogs or

derivatives of the extracellular domain of the TNF receptor. In a similar fashion, the self-associating intracellular domain (IC) or death domain (DD) thereof of the p55-R receptor or the Fas/APO1 receptor used in the oligomeric TNF receptors of the invention, may also be analogs or derivatives thereof i.e. may be any modification of the p55-IC sequence or portions thereof including the death domain (p55DD), or any modification of the Fas/APO1 intracellular domain (FAS-IC) sequence or portions thereof including the death domain (FAS DD), providing that these modifications yield a self-associating product.

Similarly, once produced and purified, the soluble oligomeric TNF receptors, analogs or derivatives thereof, may be further modified by standard chemical means to provide salts and functional derivatives thereof for the purposes of preparing pharmaceutical compositions containing as active ingredients these TNF receptors of the invention.

For the production of the soluble, oligomeric TNF receptors of the invention, the DNA sequences encoding the extracellular domain of the TNF receptor are obtained from existing clones of the entire TNF receptor, as is the intracellular domain or death domain thereof, and as is also the intracellular domain or death domain of the Fas/APO1 receptor (see Example 2 and Example 5). In this way the DNA sequence of the desired extracellular domain is ligated to the DNA sequence of the desired intracellular domain or portion thereof including the death domain, and this fused product is inserted (and ligated) into a suitable expression vector under the control of the promoter and other expression control sequences. Once formed, the expression vector is introduced (transformation, transfection, etc.) into a suitable host cell, which then expresses the vector to yield the fusion product of the invention being the soluble self-associating TNF receptor molecules. These are then purified from the host cells by standard procedures to yield the final product being the soluble, oligomeric TNF receptors.

The preferred preparation of the fusion product encoding the extracellular domain and intracellular domain or portion thereof is by way of PCR technology using oligonucleotides specific for the desired sequences to be copied from the clones encoding the entire TNF receptor molecule. Other means are also possible, such as isolating the desired portions encoding the extracellular domain and the intracellular domain, by restriction endonucleases and then splicing these together in a known fashion, with or without modifications at the terminal ends of the restriction fragments to ensure correct fusion of the desired portions of the receptor (extracellular and intracellular domains or portions thereof). The so-obtained fusion products are then inserted into the expression vector of choice.

In a similar fashion, the present invention also concerns soluble, oligomeric Fas/APO1 (FAS) receptors containing the extracellular domain of the Fas/APO1 receptor and the self-associating intracellular domain of the p55-R (p55-IC), the death domain thereof (p55DD), or the self-associating intracellular domain of the Fas/APO1 receptor (FAS-IC) or the death domain thereof (FAS DD), or any analogs or derivatives thereof (see above). The construction of these soluble, oligomeric FAS receptors is detailed in Example 5 herein below, using an available cloned full-length FAS receptor-encoding sequence as starting material and the appropriate oligonucleotides for PCR production of the desired extracellular and intracellular domains,

followed by ligation thereof to yield a fusion product, which is then inserted into a suitable expression vector. As detailed above and below, prokaryotic or eukaryotic vectors and host cells may be used to produce the desired soluble, oligomeric FAS receptors, which can then be purified and formulated, as active ingredient, into a pharmaceutical composition.

The above soluble, oligomeric FAS receptors of the invention are intended for effective blocking of the Fas ligand, which may also exist as a trimer (similar to TNF, see above), each oligomeric receptor of the invention capable of binding two or possible more Fas ligands and thereby neutralize their activity. The Fas ligand is known to be predominantly cell-surface associated but may also exist in a soluble form. In any event, the oligomeric FAS receptors of the invention can bind to at least two monomers of this ligand and thereby neutralize more effectively (than monomeric FAS receptors) the activity of the Fas ligand. The Fas ligand, and hence activation thereby of the FAS receptor, has been implicated in a number of pathological states, particularly those relating to liver damage (apoptosis of hepatocytes, for example), including liver damage associated with hepatitis, as well as in autoimmune conditions, including lymphocyte damage (apoptosis) in HIV-infected humans (see, for example Ogasawara et al., 1993, Cheng et al., 1994). Accordingly, the soluble, oligomeric FAS receptors of the invention are intended for blocking the activity of Fas ligand and may be used as active ingredient in pharmaceutical compositions for treating such Fas ligand-associated pathological states.

Likewise, the present invention also concerns soluble, oligomeric receptors which have binding affinity for both TNF and FAS-R ligand, the so-called "mixed" TNF-R/FAS-R oligomeric receptors. These mixed oligomeric receptors will contain at least one TNF-R extracellular domain and at least one FAS-R extracellular domain which are associated in the oligomeric receptor by virtue of each of these extracellular domains being fused to any one of the above-mentioned, self-associating, p55IC, p55DD, FAS IC or FAS DD

These mixed oligomeric receptors may be prepared by : (a) providing any of the above noted fusion products which contain the extracellular domain of a TNF-R (p75 TNF-R, or preferably, p55 TNF-R) fused to any one of the self-associating intracellular domains p55 IC and FAS IC or any one of the self-associating 'death domain' p55DD and FAS DD, or any self-associating portions, analogs or derivatives of any thereof; (b) providing any of the above noted fusion products which contain the extracellular domain of FAS-R fused to any one of the self-associating p55IC, FAS-IC, p55DD, and FAS DD, or any self-associating portions, analogs or derivatives of any thereof; and (c) mixing any of the TNF-specific fusion products of (a) with any of the FAS-R ligand-specific fusion products of (b) to provide (following standard selection and purification procedures) oligomeric (dimeric or higher order oligomeric) receptors which have at least both the extracellular domains of a TNF-R and FAS-R that are associated by virtue of the self-association capability of their fused IC or DD regions.

Another possibility for the preparation of the above mixed oligomeric receptors is by co-transforming suitable host cells with the above-mentioned expression vectors, one of which encodes the TNF-specific TNF-R fusion products and one of which encodes the FAS-R ligand-specific FAS-R fusion products. Following the expression of these different fusion products in the

host cells, the mixed oligomeric (TNF-R/FAS-R) receptors may be obtained by standard purification and selection procedures.

The utility of these mixed affinity oligomeric receptors is primarily for the neutralization of both TNF and FAS-R ligand when these are over-expressed endogenously or are at undesirably high levels following exogenous administration. Recent evidence points to a likelihood that there exists a synergism in function between the FAS-R ligand (usually cell-surface associated) and TNF-α (which may also be cell-surface associated). Accordingly, in some instances it is desired to neutralize both of these ligands at the same point on the cell surface, i.e. such a mixed-affinity receptor can block both the TNF binding to its receptor and the binding of FAS-R ligand to its receptor. Accordingly, these mixed-affinity receptors may be used as an active ingredient in pharmaceutical compositions for treating such conditions (see above) where both TNF and FAS-R ligand effects are undesirable.

Similarly, along the lines mentioned above concerning the soluble, oligomeric TNF-R and FAS-R, and mixed TNF-R/FAS-R oligomers of the invention, it is also possible to produce soluble, oligomeric receptors for other receptors, or any mixtures thereof, in particular those of any of the other members of the TNF/NGF super family. In this case, any of the extracellular domains of the various receptors can be fused to the above-mentioned self-associating intracellular domains or portions thereof or to any other intracellular domains of the super family members also capable of self-association.

Expression of any of the recombinant proteins of the invention as mentioned herein can be effected in eukaryotic cells (e.g. yeast, insect or mammalian cells), using the appropriate expression vectors. Any method known in the art may be employed.

For example, the DNA molecules coding for the proteins obtained by any of the above methods are inserted into appropriately constructed expression vectors by techniques well known in the art (see Sambrook et al., 1989). Double-stranded cDNA is linked to plasmid vectors by homopolymeric tailing or by restriction linking involving the use of synthetic DNA linkers or blunt-ended ligation techniques. DNA ligases are used to ligate the DNA molecules and undesirable joining is avoided by treatment with alkaline phosphatase.

In order to be capable of expressing the desired protein, an expression vector should comprise also specific nucleotide sequences containing transcriptional and translational regulatory information linked to the DNA coding for the desired protein in such a way as to permit gene expression and production of the protein. First, in order for the gene to be transcribed, it must be preceded by a promoter recognizable by RNA polymerase, to which the polymerase binds and thus initiates the transcription process. There are a variety of such promoters in use, which work with different efficiencies (strong and weak promoters). They are different for prokaryotic and eukaryotic cells.

The promoters that can be used in the present invention may be either constitutive, for example the int promoter of bacteriophage λ, the bla promoter of the β-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene of pPR325, etc., or inducible, such as the prokaryotic promoters including the major right and left promoters of

bacteriophage Δ (P_L and P_R), the trp, recA, lacZ, lacI, ompF, and gal promoters of *E. coli*, or the trp-lac hybrid promoter, etc. (Glick, B.R. (1987)). Besides the use of strong promoters to generate large quantities of mRNA, in order to achieve high levels of gene expression in prokaryotic cells, it is necessary to use also ribosome-binding sites to ensure that the mRNA is efficiently translated. One example is the Shine-Dalgarno sequence (SD sequence) appropriately positioned from the initiation codon and complementary to the 3'-terminal sequence of 16S RNA.

For eukaryotic hosts, different transcriptional and translational regulatory sequences may be employed, depending on the nature of the host. They may be derived from viral sources, such as adenovirus, bovine papilloma virus, Simian virus, or the like, where the regulatory signals are associated with a particular gene which has a high level of expression. Examples are the TK promoter of Herpes virus, the SV40 early promoter, the yeast gal4 gene promoter, etc. Transcriptional initiation regulatory signals may be selected which allow for repression and activation. so that expression of the genes can be modulated.

The DNA molecule comprising the nucleotide sequence coding for the fusion product proteins of the invention is inserted into a vector having the operably linked transcriptional and translational regulatory signals which is capable of integrating the desired gene sequences into the host cell. The cells which have been stably transformed by the introduced DNA can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may provide for phototrophy to an auxotropic host, biocide resistance, e.g. antibiotics, or heavy metals, such as copper, or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of proteins of the invention. These elements may include transcription promoters, enhancers, and termination signals. cDNA expression vectors incorporating such elements include those described by Okayama, H. (1983).

In a preferred embodiment, the introduced DNA molecule will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Factors of importance in selecting a particular plasmid or viral vector include : the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Preferred prokaryotic vectors include plasmids such as those capable of replication in *E. coli*, for example, pBR322, ColEl, pSC101, pACYC 184, etc. (see Maniatis et al., 1982; Sambrook et al., 1989); Bacillus plasmids such as pC194, pC221, pT127, etc. (Gryczan, T., (1982)); Streptomyces plasmids including pIJ101 (Kendall, K.J. et al., (1987)); Streptomyces bacteriophages such as ØC31 (Chater, K.F. et al., in : Sixth International Symposium on Actinomycetales Biology, (1986)), and Pseudomonas plasmids (John, J.F. et al., (1986), and Izaki, K. (1978)). Preferred eukaryotic plasmids include BPV, vaccinia, SV40, 2-micron circle, etc., or their derivatives. Such plasmids are well known in the art (Botstein, D. et al., (1982);

Broach, J.R. in : The Molecular Biology of the Yeast Saccharomyces : Life Cycle and Inheritance (1981); Broach, J.R., (1982); Bollon, D.P. et al., (1980); Maniatis, T., in : Cell Biology : A Comprehensive Treatise, Vol. 3 : Gene Expression, (1980); and Sambrook et al., 1989).

Once the vector or DNA sequence containing the construct(s) has been prepared for expression, the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means : transformation, transfection, conjugation, protoplast fusion, electroporation, calcium phosphate-precipitation, direct microinjection, etc.

Host cells to be used in the invention may be either prokaryotic or eukaryotic. Preferred prokaryotic hosts include bacteria such as *E. coli*, Bacillus, Streptomyces, Pseudomonas, Salmonella, Serratia, etc. The most preferred prokaryotic host is *E. coli*. Bacterial hosts of particular interest include *E. coli* K12 strain 294 (ATCC 31446), *E. coli* X1776 (ATCC 31537), *E. coli* W3110 (F^-, lambda$^-$, prototropic (ATCC 27325)), and other enterobacterium such as Salmonella typhimurium or Serratia marcescens and various Pseudomonas species. Under such conditions, the protein will not be glycosylated. The prokaryotic host must be compatible with the replicon and control sequences in the expression plasmid.

Preferred eukaryotic hosts are mammalian cells, e.g. human, monkey, mouse and Chinese hamster ovary (CHO) cells, because they provide post-translational modifications to protein molecules including correct folding or glycosylation at correct sites. Also yeasts cells can carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides).

After the introduction of the vector, the host cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the desired proteins.

Purification of the recombinant proteins is carried out by any one of the methods known for this purpose, i.e. any conventional procedure involving extraction, precipitation, chromatography, electrophoresis, or the like. A further purification procedure that may be used in preference for purifying the protein of the invention is affinity chromatography using anti-TNF receptor monoclonal antibodies, which are produced and immobilized on a gel matrix contained within a column. Impure preparations containing the recombinant protein are passed through the column. The protein will be bound to the column by the specific antibody while the impurities will pass through. After washing, the protein is eluted from the gel by a change in pH or ionic strength.

As used herein (see above), the term 'salts' refers to both salts of carboxyl groups and to acid addition salts of amino groups of the protein molecule formed by means known in the art. Salts of a carboxyl group include inorganic salts, for example, sodium, calcium, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or

lysine. Acid addition salts include, for example, salts with mineral acids and salts with organic acids.

"Functional derivatives" as used herein covers derivatives which may be prepared from the functional groups which occur as side chains on the residues or the N- or C- terminal groups, by means known in the art, and are included in the invention as long as they remain pharmaceutically acceptable, i.e. they do not destroy the activity of the protein and do not confer toxic properties on compositions containing it. These derivatives include aliphatic esters or amides of the carboxyl groups, and N-acyl derivatives of free amino groups of O-acyl derivatives of free hydroxyl groups formed with acyl moieties (e.g. alkanoyl or carbocyclic aroyl groups).

"Fractions" as used herein refers to any part or portion of the receptor, (intracellular or extracellular domains thereof), or of the proteins binding to the intracellular domain of the receptor, provided it retains its biological activity.

As mentioned above, the present invention also relates to various pharmaceutical compositions comprising a pharmaceutically acceptable carrier and the various noted active ingredients of the invention or their salts, functional derivatives, or mixtures of any of the foregoing. These compositions may be used in any of the conditions as noted herein, for example, in conditions where there is an over production of endogenous TNF, such as in cases of septic shock, cachexia, graft-versus host reactions, autoimmune diseases like rheumatoid arthritis, etc. The way of administration can be via any of the accepted modes of administration for similar agents and will depend on the condition to be treated, e.g. when used to inhibit TNF effects they may be administered intravenously in case of septic shock or local injection in case of rheumatoid arthritis (for example, into the knee), or continuously by infusion, etc. The compositions may also be used, for example, in cases of TNF intoxication caused by exogenous administration of excessive amount (overdoses) of TNF, e.g. in the case of cancer therapy or viral disease therapy.

The pharmaceutical compositions of the invention are prepared for administration by mixing the protein or its derivatives with physiologically acceptable carriers, stabilizers and excipients, and prepared in dosage form, e.g. by lyophilization in dosage vials. The amount of active compound to be administered will depend on the route of administration, the disease to be treated and the condition of the patient. For example, local injection in case of inflammatory conditions of rheumatoid arthritis will require less active ingredient on a body weight basis than will intravenous infusion in case of septic shock.

Other aspects of the invention will be apparent from the following examples.

The invention will now be described in more detail in the following non-limiting examples and the accompanying drawings :

EXAMPLE 1

Cloning and isolation of proteins which bind to the intracellular domains of the p55 and p75 TNF receptors

To isolate proteins interacting with the intracellular domains of the p55 and p75 TNF receptors (p55IC and p75 IC), the yeast two-hybrid system was used (Fields and Song, 1989).

Briefly, this two-hybrid system is a yeast-based genetic assay to detect specific protein-protein interactions *in vivo* by restoration of a eukaryotic transcriptional activator such as GAL4 that has two separate domains, a DNA binding and an activation domain, which domains when expressed and bound together to form a restored GAL4 protein, is capable of binding to an upstream activating sequence which in turn activates a promoter that controls the expression of a reporter gene, such as lacZ or HIS3, the expression of which is readily observed in the cultured cells. In this system the genes for the candidate interacting proteins are cloned into separate expression vectors. In one expression vector the sequence of the one candidate protein is cloned in phase with the sequence of the GAL4 DNA-binding domain to generate a hybrid protein with the GAL4 DNA-binding domain, and in the other vector the sequence of the second candidate protein is cloned in phase with the sequence of the GAL4 activation domain to generate a hybrid protein with the GAL4-activation domain. The two hybrid vectors are then co-transformed into a yeast host strain having a lacZ or HIS3 reporter gene under the control of upstream GAL4 binding sites. Only those transformed host cells (cotransformants) in which the two hybrid proteins are expressed and are capable of interacting with each other, will be capable of expression of the reporter gene. In the case of the lacZ reporter gene, host cells expressing this gene will become blue in color when X-gal is added to the cultures. Hence, blue colonies are indicative of the fact that the two cloned candidate proteins are capable of interacting with each other.

Using this two-hybrid system, the intracellular domains p55IC and p75IC were cloned, separately, into the vector pGBT9 (carrying the GAL4 DNA-binding sequence, provided by CLONTECH, USA, see below), to create fusion proteins with the GAL4 DNA-binding domain (similarly, the intracellular domain, FAS-IC and a portion of the 55IC, namely, the 55DD were also cloned into pGBT9 and used to isolate other IC-binding proteins, see Example 3 below). For the cloning of p55IC and p75IC into pGBT9, clones encoding the full-length cDNA sequences of p55 TNF-R (Schall et al., 1990) and p75 TNF-R (Smith et al., 1990) were used from which the intracellular domains (IC) were excised as follows : p55IC was excised using the enzymes EcoRI and SalI, the EcoRI-SalI fragment containing the p55IC sequence was then isolated by standard procedures and inserted into the pGBT9 vector opened, in its multiple cloning site region (MCS), with EcoRI and SalI. p75 IC was excised using the enzymes BspHI and SalI, the BspHI-SalI fragment containing the p75 IC sequence was then isolated by standard procedures and filled-in with the Klenow enzyme to generate a fragment which could be inserted into the pGBT9 vector opened with SmaI and SalI.

The above hybrid (chimeric) vectors were then cotransfected (separately, one cotransfection with the p55IC hybrid and one with the p75 IC hybrid vector) together with a cDNA library from human HeLa cells cloned into the pGAD GH vector, bearing the GAL4 activating domain, into the HF7c yeast host strain (all the above-noted vectors, pGBT9 and pGAD GH carrying the HeLa cell cDNA library, and the yeast strain were purchased from Clontech Laboratories, Inc., USA, as a part of MATCHMAKER Two-Hybrid System, #PT1265-1). The co-transfected yeasts were selected for their ability to grow in medium lacking Histidine (His^- medium), growing colonies being indicative of positive transformants. The selected yeast

clones were then tested for their ability to express the lacZ gene, i.e. for their LAC Z activity, and this by adding X-gal to the culture medium which is catabolized to form a blue colored product by β-galactosidase, the enzyme encoded by the lacZ gene. Thus, blue colonies are indicative of an active lacZ gene. For activity of the lacZ gene, it is necessary that the GAL4 transcription activator be present in an active form in the transformed clones, namely that the GAL4 DNA-binding domain encoded by one of the above hybrid vectors be combined properly with the GAL4 activation domain encoded by the other hybrid vector. Such a combination is only possible if the two proteins fused to each of the GAL4 domains are capable of stably interacting (binding) to each other. Thus, the His^+ and blue (LAC Z^+) colonies that were isolated are colonies which have been cotransfected with a vector encoding p55IC and a vector encoding a protein product of human HeLa cell origin that is capable of binding stably to p55 IC; or which have been transfected with a vector encoding p75IC and a vector encoding a protein product of human HeLa cell origin that is capable of binding stably to p75 IC.

The plasmid DNA from the above His^+, LAC Z^+ yeast colonies was isolated and electroporated into E. coli strainHB101 by standard procedures followed by selection of Leu^+ and Ampicillin resistant transformants, these transformants being the ones carrying the hybrid pGAD GH vector which has both the Amp^R and Leu^2 coding sequences. Such transformants therefore are clones carrying the sequences encoding newly identified proteins capable of binding to the p55IC or p75IC. Plasmid DNA was then isolated from these transformed E. coli and retested by :

(a) retransforming them with the original intracellular domain hybrid plasmids (hybrid pGTB9 carrying either the p55IC or p75IC sequences) into yeast strain HF7 as set forth hereinabove. As controls, vectors carrying irrelevant protein encoding sequences, e.g. pACT-lamin or pGBT9 alone were used for cotransformation with the p55IC-binding protein or p75IC-binding protein encoding plasmids. The cotransformed yeasts were then tested for growth on His^- medium alone, or with different levels of 3-aminotriazole; and

(b) retransforming the plasmid DNA and original intracellular domain hybrid plasmids and control plasmids described in (a) into yeast host cells of strain SFY526 and determining the LAC Z^+ activity (effectivity of β-gal formation, i.e. blue color formation).

The results of the above tests revealed that the pattern of growth of colonies in His^- medium was identical to the pattern of LAC Z activity, as assessed by the color of the colony, i.e. His^+ colonies were also LAC Z^+. Further, the LAC Z activity in liquid culture (preferred culture conditions) was assessed after transfection of the GAL4 DNA-binding and activation-domain hybrids into the SFY526 yeast hosts which have a better LAC Z inducibility with the GAL4 transcription activator than that of the HF-7 yeast host cells.

The results of the above co-transfections are set forth in Table 1 below, from which it is apparent that a number of proteins were found that were capable of binding to the p55IC or the p75IC, namely, the proteins designated 55.11, which binds to the p55IC; and 75.3 and 75.16 which bind to the p75IC. All of these p55IC- and p75IC-binding proteins are authentic human proteins all encoded by cDNA sequences originating from the HeLa cell cDNA library, which

were fused to the GAL4 activation-domain sequence in the plasmid pGAD GH in the above yeast two-hybrid analysis system

Interestingly, it was also found that fragments of the p55IC, itself, namely, the proteins designated 55.1 and 55.3 were capable of binding to p55IC. These are discussed also in Example 2 below.

TABLE 1

SUMMARY OF THE CHARACTERISTICS OF SOME OF THE cDNA CLONES (SEE ALSO EXAMPLE 3) ISOLATED BY THE TWO-HYBRIDSYSTEM APPROACH

DNA-binding domain hybrid	Activation-domain hybrid	Colony color	Lac Z activity in liquid culture assay
pGBT9-IC55	---	white	0.00
pGBT9-IC55	55.1	blue	0.65
pGBT9-IC55	55.3	blue	0.04
---	55.1	white	0.00
---	55.3	white	0.00
pACT-Lamin	55.1	white	0.00
pACT-Lamin	55.3	white	0.00
pGBT9	55.1	white	0.00
pGBT9	55.3	white	0.00
pGBT9-IC55	55.11	blue	ND
---	55.11	white	ND
pACT-Lamin	55.11	white	ND
pGBT9	55.11	white	ND
pGBT9-IC75	75.3	blue	ND
pGBT9-IC75	---	white	ND
---	75.3	white	ND
pACT-Lamin	75.3	white	ND
pGBT9	75.3	white	ND
pGBT9-IC75	75.16	blue	ND
---	75.16	white	ND
pACT-Lamin	75.16	white	ND
pGBT9	75.16	white	ND

In the above Table 1, the plasmids and hybrid encoding the GAL4 DNA-binding domain and GAL4 activation domain are as follows :

DNA-binding domain hybrids :

pGBT9-IC55 : full-length intracellular domain of the p55-TNF-R (p55IC)

pACT-Lamin : irrelevant protein - lamin.

pGBT9 : vector alone

pGBT9-IC75 : full-length intracellular domain of the p75-TNF-R (p75IC)

Activation-domain hybrid :

55.1 and 55.3 correspond to fragments of the intracellular domain of the p55-TNF-R.

55.11 : is the novel protein associating with the p55-TNF-R

75.3 and 75.16 are the novel proteins associating with the p75-TNF-R.

The above noted cloned cDNAs encoding the novel p55IC- and p75IC- binding proteins, 55.11, 75.3 and 75.16, were then sequenced using standard DNA sequencing procedures. The partial sequence of all of these protein-encoding sequences is set forth in Figs. 1 a-c, where Fig. 1(a) depicts the sequence of the cDNA encoding protein 55.11; Fig. 1(b) depicts the partial sequence of the cDNA encoding protein 75.3; and Fig. 1(c) depicts the partial sequence of the cDNA encoding protein 75.16. In Fig. 1(d) there is shown the deduced amino acid sequence of the protein 55.11, as deduced from the nucleotide sequence of Fig. 1(a).

It should be noted, however, that a partial sequence of the cDNA encoding the 55.11 protein has also been reported by Khan et al. (1992), in a study of human brain cDNA sequences, which study was directed at the establishment of a new rapid and accurate method for the sequencing and physical and genetic mapping of human brain cDNAs. However, Khan et al. did not provide any information as regards the function or any other characteristics of the protein encoded by the 55.11 cDNA sequence, such functional or other analysis not being the intention of Khan et al. in their study.

Analysis and characterization of the 55.11 protein

(a) General Procedures and Materials

(i) Cloning of the cDNA of 55.11

Upon the analysis (for example, Northern Analysis -see below) of the cDNA of protein 55.11, it was revealed that the above noted 55.11 cDNA cloned by the two-hybrid screen procedure represented only a partial cDNA of 55.11 having nucleotides 925-2863 (see Fig. 1(a)) which code for amino acids 309-900 (see Fig. 1(d)). The remaining part of the 55.11 cDNA [nucleotides 1-924 (Fig. 1(a)) which code for amino acids 1-308 (Fig. 1(d))] was obtained by standard procedures, namely, by cloning by PCR from a human fetal liver cDNA library (for more details, see below). The full nucleotide sequence of 55.11 (Fig. 1(a)) was determined in both directions by the dideoxy chain termination method.

(ii) Two-hybrid β-galactosidase expression tests

β-galactosidase expression tests were performed as described above, except that in some of the tests, the pVP16 vector, which contains the activation domain of VP16, was used instead of pGAD-GH, the Gal4 activation domain vector. Numbering of residues in the proteins encoded by the cDNA inserts are as in the Swiss-Prot data bank. Deletion mutants were produced by PCR, and point mutations by oligonucleotide-directed mutagenesis (Kunkel, 1994).

(iii) Northern analysis

Total RNA was isolated using TRI REAGENT (Molecular Research Center, Inc., Cincinnati, Oh., U.S.A.), denatured in formaldehyde/formamide buffer, electrophoresed through an agarose/formaldehyde gel, and blotted to a GeneScreen Plus membrane (Dupont, Wilmington, De., U.S.A.) in 10xSSPE buffer, using standard techniques. The blots were hybridized with the partial cDNA of 55.11 (see above, nucleotides 925-2863), radiolabeled with the random-prime kit (Boehringer Mannheim Biochemica, Mannheim, Germany), and washed stringently. Autoradiography was performed for 1 week.

(iv) Expression of 55.11 cDNA in HeLa cells and binding of the 55.11 protein to glutathione S-transferase fusion proteins f p55-IC

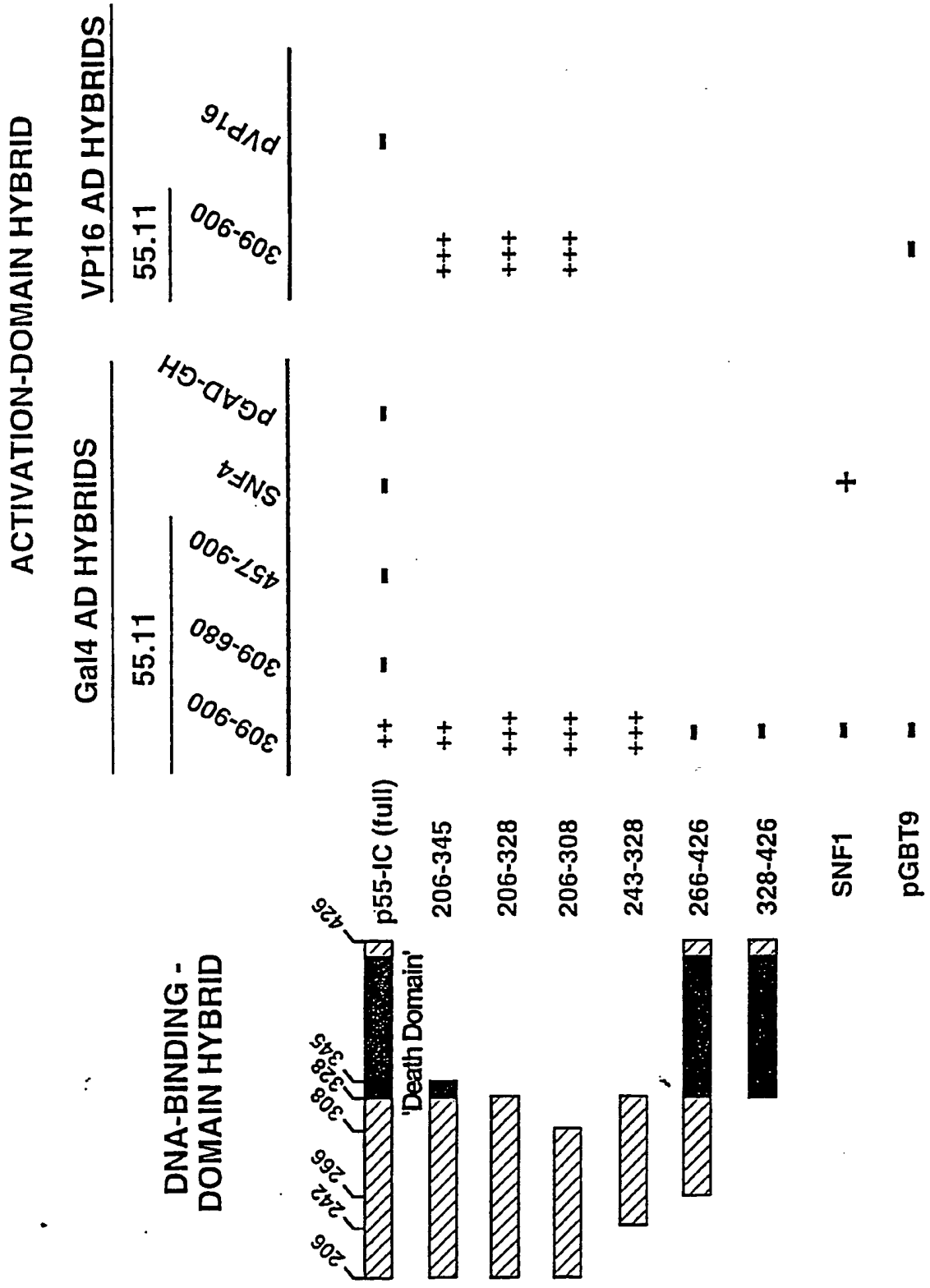
Glutathione S-transferase (GST) fusions with p55-IC (GST-p55IC) and with p55-IC truncated below amino acid 345 (GST-p55IC345) were produced and adsorbed to glutathione-agarose beads as described in Example 2 below (see also Smith and Corcoran, 1994; Frangioni and Neel, 1993). The cDNAs of 55.11 (1-2863 nucleotides, i.e. the full-length 55.11 cDNA), of FLAG-55.11, and of luciferase were expressed in HeLa cells. FLAG-55.11 is the region extending between residues 309 and 900 in the 55.11 protein (the partial cDNA of 55.11 (nucleotides 925-2863), originally cloned by the two hybrid screen), N-linked to the FLAG octapeptide (Eastman Kodak, New Haven, Ct., U.S.A.). Expression of the fusion proteins was accomplished using a tetracycline-controlled expression vector (HtTA-1) in a HeLa cell clone that expresses a tetracycline-controlled transactivator (see Example 2 below, and Gossen and Bujard, 1992). Metabolic labeling of the expressed proteins with [^{35}S] Met and [^{35}S] Cys (Dupont, Wilmington, De., U.S.A. and Amersham, Buckinghamshire, England), lysis of the HeLa cells, immunoprecipitation, and binding of the labeled proteins to the GST fusion proteins were performed as described below (Example 2), except that 0.5% rather than 0.1% Nonidet P-40 was present in the cell lysis buffer. The immunoprecipitations of 55.11 and FLAG-55.11 were achieved using a rabbit antiserum (diluted 1:500) raised against a GST fusion protein containing the region of 55.11 that extends between amino acids 309 and 900 and a mouse monoclonal antibody against the FLAG octapeptide (M2; Eastman Kodak; 5 µg/ml of cell lysate).

(b) Binding of the 55.11 protein to p55-IC within transformed yeasts

In this study it was sought to ascertain the nature of the binding between 55.11 and p55IC, in particular, the regions of both of these proteins involved in this binding. For this purpose the above two-hybrid procedure was used in which various full-length and deletion mutants of p55IC (see also Example 2 below) in "DNA-binding domain" constructs were used as "baits" to bind the "preys", being the partial 55.11 protein encoded in constructs in which the partial 55.11 sequence (residues 309-900, as originally isolated) was fused to the "activation domain" in the vectors GAL4AD and VP16AD. Further, various deletion mutants of 55.11 were also constructed and fused to the "activation domain" in the GAL4AD vector (e.g. mutants of 55.11 having only residues 309-680 and 457-900). The binding of the various 'binding domain' constructs to the various 'activation domain' constructs was examined in transfected SFY526 yeast cells. The binding was assessed by a two-hybrid β-galactosidase expression filter assay. The non-relevant proteins SNF1 and SNF4 served as positive controls for the 'binding domain' and 'activation domain' constructs, respectively; the empty Gal4 (pGAD-GH) and VP16 (pVP16) vectors served as negative controls for the 'activation domain' constructs; and the empty Gal4 (pGBT9) vector served as a negative control for the 'binding domain' constructs. The results of the assay are set forth in Table 2 below in which the symbols "+++" and "++" indicate the development of strong color within 20-60 min of initiation of the assay, respectively (positive binding results); and "-" indicates no development of color within 24h of commencement of the assay (negative results). Blank spaces in the Table indicate binding assays not tested.

Table 2

Binding of the 55.11 protein to p55-IC
within transformed yeasts



From the results presented in Table 2 above it may be concluded that 55.11 binds to p55-IC at a site which is distinct from the 'death domain' (residues 328-426) of p55-IC.

The 55.11 protein bound to a truncated p55-IC from which the death domain had been deleted (construct 206-328 in Table 2), more effectively than to nontruncated p55-IC. It also bound to an even further C terminally truncated construct (construct 206-308) and to a construct from which both the death domain and a membrane proximal part were deleted (construct 243-328). However, the 55.11 protein did not bind to a construct that was N-terminally truncated down to amino acid 266 (Table 2). These findings indicate that the binding site for 55.11 is located in the region that extends between residues 243 and 308 of p55-IC and that the N terminus of this binding site is between residues 243 and 266.

Transfer of the cDNA for 55.11 from the originally cloned 'prey' construct, which contained the Gal4 activation domain, to a prey construct containing the VP16 activation domain did not decrease the binding efficiency of the 55.11 protein to p55-IC (Table 2). Thus, the structure(s) involved in this binding appear to reside within the 55.11 molecule and not to involve the site of fusion of 55.11 with the activation domain.

However, binding of 55.11 to p55-IC was abolished by even limited truncations of the 55.11 protein at either its C (55.11 construct 309-680) or N terminus (55.11 construct 457-900). (residue 309 is the first residue in the 55.11 protein encoded by the partial cDNA clone originally isolated in the two hybrid screen).

The observed binding between 55.11 and p55-IC appeared to be specific since 55.11 did not bind to other proteins, including three receptors of the TNF/NGF receptor family (p75-R, Fas/APO1 and CD40) and other proteins such as lamin and cyclin D (data not shown). It should be noted that of the other TNF/NGF receptor proteins tested there was also tested portions thereof which include their intracellular domains : human FAS-R (residues 175-319), CD40 (residues 216-277) and p75-TNF-R (residues 287-461), none of which bound 55.11 (data not shown).

(c) Northern analysis of the RNA from several cell lines, using the 55.11 cDNA as a probe and cloning of the full-length 55.11 cDNA

The cell lines examined were HeLa, CEM, Jurkat, and HepG2 cells derived from human epithelial carcinoma, an acute lymphoblastic T cell leukemia, an acute T cell leukemia, and a hepatocellular carcinoma, respectively. The 55.11 cDNA original isolated (nucleotides 925-2863) was used as a probe. Samples consisted of 10 μg of RNA/lane. The results of the Northern analysis are shown in Fig. 2, which is a reproduction of a Northern blot.

From Fig. 2 it is thus apparent that the Northern analysis using the 55.11 cDNA as a probe revealed, in several cell lines, a single hybridizing transcript of about 3 kB, which is larger than the cDNA (2 kB) of the originally isolated 55.11 cDNA. Using oligonucleotide primers that correspond to the 55.11 sequence, we cloned by PCR a 5' extending sequence whose length was about 1 kB. The sum of the length of this 5' extending sequence with that of the originally cloned cDNA approximates the length of the 55.11 transcript. The 3 kB cDNA that encompassed both

these portions was effectively expressed in transfected HeLa cells (see below) yielding a protein of about 84 kDa, which suggests that the 3 kB cDNA contains a translational start site.

(d) *In vitro* binding of the 55.11 protein to GST-fusion proteins containing portions of p55-IC

To ascertain that 55.11 can indeed bind to p55-IC and to exclude involvement of yeast proteins in this binding, the *in vitro* interaction of GST p55-IC fusion proteins, produced by bacteria, with the protein encoded by the 3 kB 55.11 cDNA (55.11-full), produced by transfected HeLa cells, was examined. In this study the cDNAs for the full-length 55.11, FLAG-55.11 (residues 309-900 of 55.11 encoded by the originally cloned partial cDNA and fused at the N terminus with the FLAG octapeptide), and luciferase (control) were expressed in transfected HeLa cells and metabolically labeled with [^{35}S] Met and [^{35}S] Cys. The following proteins were fused with GST : full-length p55-IC (GST-p55-IC) and p55-IC C-terminally truncated up to amino acid 345 (GST-p55-IC345) to remove most of the 'death domain' (see Table 2). GST alone served as a control. Lysates of the transfected cells were immunoprecipitated with antibodies against the 55.11 protein when the full-length 55.11 protein was used for binding the GST-fusion proteins, or with antibodies against the FLAG octapeptide when the FLAG-55.11 fusion product was used for binding the GST-fusion proteins. The proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 10% acrylamide), followed by autoradiography.

In Figs. 3A and B are shown reproductions of the autoradiograms of the above SDS-PAGE gels, in which Fig. 3A depicts the binding of the full-length 55.11 protein (55.11-full) to the various GST-fusion proteins; and in which Fig 3B depicts the binding of the Flag-55.11 fusion product to the various GST-fusion proteins. In Fig. 3A there is shown in the extreme right hand lane a control immunoprecipitate of lysates of cells transfected with only the full-length 55.11 and immunoprecipitated with the anti-55.11 antibodies (α55.11 Abs). In Fig. 3B there is shown in the extreme right hand lane a control immunoprecipitate of lysates of cells transfected with only the FLAG-55.11 and immunoprecipitated with the anti-FLAG antibodies (αFLAG Abs).

Thus, it is apparent from Figs. 3A and B that the protein encoded by the full-length 55.11 cDNA can be expressed in HeLa cells and it binds to fusion proteins that contained the full p55-IC (GST-p55IC) or a truncated p55-IC that lacked most of the death domain (GST-p55IC345) (Fig. 3A). The full-length 55.11 protein did not bind to GST alone (control). Similarly, the HeLa cell-expressed protein encoded by the initially cloned partial cDNA of 55.11 in fusion with the FLAG octapeptide (FLAG-55.11) bound *in vitro* to GST-p55IC and GST-p55IC345, but not to GST (Fig. 3B). The above results also therefore provide additional evidence (see (b) above) that the 55.11 binds to a region of the p55IC upstream of the 'death domain', i.e. in the region of the p55-IC that is more proximal to the transmembrane domain.

Moreover, the above study also demonstrates that, in accordance with the present invention, antibodies to 55.11 have been successfully produced (Fig. 3A).

(e) Comparison of the deduced amino acid sequence of human 55.11 to that of related proteins present in lower organisms, and sequence features of the 55.11 protein

As mentioned above, in accordance with the present invention, the full-length 55.11 cDNA has been cloned and sequenced (see nucleotide sequence in Fig. 1(a)) and the full amino acid sequence of 55.11 has been deduced from the cDNA sequence (see amino acid sequence in Fig. 1(d)). Data bank (GenBank™/EMBL DataBank) searches revealed that parts of the sequence of the human 55.11 cDNA (accession numbers T03659, Z19559, and F09128) and its mouse homologue (accession numbers X80422 and Z31147) have already been determined during arbitrary sequencing of cDNA libraries. A cDNA sequence (accession number U18247) that encodes for a human protein of 596 amino acids present in cultures of human hepatoma HC10 cells is similar to that of 55.11. This hepatoma protein, however, lacks an N terminal portion (amino acids 1-297) corresponding to that of 55.11 and also differs from 55.11 at the regions that correspond to residues 297-377 and residues 648-668 in 55.11. The searches of the data bank also revealed that proteins with very high sequence homology to 55.11 exist in *Saccharomyces cerevisiae* (yeasts), *Arabidopsis thaliana* (plants) and *Caenorhabditis elegans* (worms). Thus, 55.11 appears to fulfill an evolutionary conserved function. In the yeasts, there are two known proteins (the open reading frame YHR027c and SEN3) whose DNA sequences resemble that of 55.11. The sizes of both are close to that of 55.11. YHR027c is known only by the sequencing of a genomic clone while SEN3 has been cloned as a cDNA. The sites within 55.11 that are similar to those in SEN3 correlate to the sites of its similarity to YHR027c, although much more similarity is evident between 55.11 and YHR027c than between 55.11 and SEN3. The DNA sequence information available for the *Arabidopsis thaliana* and *Caenorhabditis elegans* proteins, although only partial, clearly shows that these proteins are as similar to 55.11 as the YHR027c protein of yeast. The only one of these four proteins whose nature has been elucidated so far is the yeast SEN3, whose homology to 55.11 is limited. SEN3 has been identified as the yeast equivalent of the p112 subunit of an activator of the 20S proteasome (the proteolytic core of the 26S proteasome [Rechsteiner et al., 1993; DeMartino et al., 1994]) (M.R. Culbertson and M. Hockstrasser, personal communication).

In Fig. 4 there is shown schematically a comparison of the deduced amino acid sequence of human 55.11 to that of the above-mentioned, related proteins present in lower organisms. In Fig. 4 the sequences that are compared are the sequences of amino acids predicted for : the 55.11 cDNA (see Fig. 1(d)); an open reading frame (YHR027c) within a cosmid derived from the 8th chromosome of *Saccharomyces cerevisiae* (nucleotides 21253-24234, accession number U10399); SEN3, the cDNA of a *Saccharomyces cerevisiae* protein (accession number L06321); a partial cDNA of a protein of the plant *Arabidopsis thaliana* (accession number T21500); and a partial cDNA of a protein of the nematode *Caenorhabditis elegans* (accession number D27396). The 'KEKE' sequence in 55.11 is marked with a solid line and the sequence AYAGS$(x)_8$LL with broken lines. The sequences were aligned using the PILEUP and PRETTYBOX programs of the GCG package. Gaps introduced to maximize alignments are denoted by dashes.

As regards the various sequence features or motifs present in the human 55.11 sequence the following has been observed : Conserved amino acid sequence motifs were not discerned within the protein encoded for by 55.11, except for a repetitive 'KEKE' sequence that extends between Lys 614 and Glu 632 (underlined in Fig. 4). Such 'KEKE' sequences, which are present in many proteins, including proteasonal subunits and chaperonins, may promote association of protein complexes (Realini et al., 1994). A sequence AYAGS(x)$_8$LL appears twice in the 55.11 protein (at sites 479 590. see Fig. 4); no functional significance for this sequence has yet been described.

(f) Sequence features of the p55IC region involved in binding to the 55.11 protein

As described above (see (b) and (d)), the 55.11 protein binds to a region of the p55-IC between residues 243 and 308 (the N terminus of this binding site being between residues 243 and 266), this region being upstream of the 'death domain' and more proximal to the transmembrane domain of the p55-TNF-R. This region within p55-IC to which 55.11 binds has a high content of proline, serine, and threonine residues. However, this region does not contain the RPM1 and RPM2 proline-rich motifs present in several other cytokine receptors (ONeal and Yu-Lee, 1993). In the region that extends between residues 243 and 266, whose deletion abolishes the binding of p55-R to 55.11 (see (b) and (d) above and Table 2), two of the serines and two of the threonines are followed by proline residues, which makes them potential sites for phosphorylation by MAP kinase, CDC2, and other proline-dependent kinases (Seger and Krebs, 1995). Phosphorylation of this site in the receptors might affect its binding to the 55.11 protein.

In view of all of the aforementioned with regards to protein 55.11 and its binding to p55-IC it can be concluded that in accordance with the present invention, a new protein has been found which binds to a distinct region upstream to the 'death domain' of p55-IC. Such binding could affect TNF-mediated activities other than induction of cell death. The region to which 55.11 binds has previously been shown to be involved in induction of nitric oxide synthase (Tartaglia et al., 1993), and appears to be involved in the activation of the neutral sphingomyelinase by TNF (Wiegmann et al., 1994). It is thus possible that association (binding) of 55.11 with the intracellular domain of p55-TNF-R (p55IC) affects or is involved in : (i) the signaling for these above noted or other TNF effects, (ii) the folding or processing of the protein (as suggested by the similarity of 55.11 to a subunit of the 26S proteasome), or (iii) the regulation of the activity or expression of p55-TNF-R.

EXAMPLE 2

Self-association ability of the intracellular domain of the p55 TNF receptor (p55IC) and its capability to cause cell death and other features and activities thereof, and a related Fas/APO1 receptor's intracellular domain

As set forth in Example 1 above, it was discovered that the intracellular domain of p55 TNF-R (p55IC) is capable of binding to itself, and further that fragments of p55IC, namely proteins 55.1 and 55.3, are also capable of binding to p55IC.

It is known that the binding of TNF to p55 TNF-R leads to a cytocidal effect on the cells carrying this receptor. Further, antibodies against the extracellular domain of this receptor can themselves trigger this effect, in correlation with the effectivity of receptor cross-linking by them.

In addition, mutational studies (Tartaglia et al., (1993); Brakebusch et al., (1992)) showed that the function of the p55-R depends on the integrity of its intracellular domain. It was therefore suggested that the initiation of signaling for the cytocidal effect of TNF occurs as a consequence of association of two or more intracellular domains of the p55-R (p55-IC), imposed by receptor aggregation. The results in accordance with the present invention provide further evidence for this notion, showing that expression of the intracellular domain of the p55-R within cells, without the transmembrane or intracellular domain, triggers their death. Such free intracellular domains of the p55-R are shown to self associate, which probably accounts for their ability to function independently of TNF. The fact that the signaling by the full length p55-R does depend on TNF stimulation is suggested to reflect activiti(es) of the transmembrane or extracellular domain of the receptor which decrease or prevent this self association.

The ability of the intracellular domain of the p55-R (p55-IC) to self associate was found serendipitously, in the attempts to clone effector proteins which interact with this receptor (see Example 1 above). We applied for that purpose the above mentioned "two hybrid" technique. In addition to the novel protein, 55.11 found to associate (bind) to the p55IC, it was also found that three other cloned HeLa cell cDNAs contained cDNA sequences encoding for parts of the intracellular domain of the p55-R, implying that the p55-IC is capable of self-association. Two of these clones were identical, containing an insert which encodes for amino acids 328-426 (designated as clone 55.1 encoding protein fragment 55.1 of the p55IC). The third contained a longer insert, encoding for amino acids 277-426 (designated as clone 55.3 encoding protein fragment 55.3 of the p55IC).

In addition, we assessed the in vitro interaction between two bacterially produced chimeras of the p55IC, one, in which it was fused to the maltose binding protein (MBP) and the other in which is was fused to the glutathione-S-transferase (GST). These chimeras were constructed, cloned and expressed by standard methods. Following their expression, the assessment of the self-interaction of the p55-R intracellular domain (p55IC) by determining the interaction of the above bacterially-produced chimeric proteins GST-IC55 (Mr - 51kD) and MBP-IC55 (Mr - 67 kD) with each other. Equal amounts of the GST-IC55 chimera (samples of lanes 1-4 in Fig. 5) or GST alone (samples of lanes 5-8 in Fig. 5) were bound to glutathione-agarose beads (Sigma) and were then incubated with the same amount of MBP-IC55 fusion protein in one of the following buffer solutions :

(i) buffer I (20mM Tris-HCl, pH 7.5, 100mM KCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 5mM DTT, 0.2% Triton X100, 0.5mM PMSF, 5% Glycerol). This was done for the samples of Lanes 1 and 5 of Fig. 5.

(ii) buffer I containing 5mM EDTA instead of $MgCl_2$. This was done for the samples of Lanes 2 and 6 of Fig. 5.

(iii) buffer I containing 250mM instead of 100mM KCl. This was done for the samples of Lanes 3 and 7 of Fig. 5.

(iv) buffer I containing 400mM instead of 100mM KCl. This was done for the samples of Lanes 4 and 8 of Fig. 5.

After incubation with rotation for 2h at 4°C, the beads were washed with the same buffers and then boiled in SDS-PAGE buffer followed by electrophoresis by PAGE. The proteins on the gel were then Western blotted to a nitrocellulose membrane which was then stained with polyclonal antiserum against MBP. A reproduction of this stained Western blot is shown in Fig. 5, the samples in lanes 1-8 being those noted above.

From Fig. 5 it is apparent that the p55IC-MBP chimera bind to the p55IC-GST chimera (lanes 1-4) independently of divalent cations and even at a rather high salt concentration (0.4M KCl). Thus, it is concluded that the p55IC is able to avidly self-associate.

To evaluate the functional implications of the propensity of the p55-IC to self associate, we attempted to express the p55-IC within the cytoplasm of cells which are sensitive to the cytocidal effect of TNF. Considering the possibility that the p55-IC will turn to be cytotoxic, we chose to express it in an inducible manner, using the recently developed, tightly regulated tetracycline-controlled mammalian expression system (Gossen and Boujard, 1992). Expression of the p55-IC resulted in massive cell death (Fig. 6, right panel). The dying cells displayed cell surface blabbing as observed in the killing of the cells by TNF. Transfection of the p55-IC construct to the cells in the presence of tetracycline, which reportedly decreases the expression of pHD10-3 regulated constructs by as much as 10^5 fold, still resulted in some cell death, although significantly less than that observed in the absence of tetracycline (Fig 6, left panel). In contrast, cells transfected with a control construct, containing the lucipherase cDNA, showed no signs of death (results not shown).

The ability of the p55-IC to trigger cell death, when expressed without the transmembrane or extracellular domains of the receptor, provides further evidence for the involvement of this domain in signaling. Furthermore, it indicates that no other part of the receptor plays a direct role in such signaling.. Studies of the effects of mutations, including those mutations studied in the present invention, on the function of the p55-IC, indicated that the region extending between amino acid residues 326 and 407 is most critical for its function. This region shows marked resemblance to sequences within the intracellular domains of two other receptors, evolutionarily related to the p55 TNF-R - namely, the Fas receptor (Itoh et al., 1991; Oehm et al., 1992), which can also signal for cell death and CD40 -a receptor (Stamenkovic et al., 1989) which enhances cell growth; this sequence therefore seems to constitute a conserved motif which plays some kind of general role in signaling. Since it does not resemble known motives characteristic of enzymatic activities, it seems plausible that it signals in indirect manner, i.e. possibly by serving as a docking site for signaling enzymes or for proteins which transmit stimulatory signals to them. The p55-IC, the Fas receptor and CD 40 can all be stimulated by antibodies against their extracellular domain. Their stimulation could be shown to correlate with the ability of the antibodies to cross-link the receptors. It therefore seems that the signaling is initiated as a consequence of interaction of two

or more intracellular domains imposed by aggregation of the extracellular domains. Involvement of such interaction in the initiation of signaling of these receptors was also indicated by studies (Brakebusch et al., 1992) showing that expression of receptors made nonfunctional by mutation of their intracellular domain, had a "dominant negative" effect on the function of co-expressed normal receptors. Aggregation of the p55-R in response to TNF was suggested to occur in a passive manner, merely due to the fact that each of the TNF molecules, which occur as homotrimers, can bind two or three receptor molecules. However, the findings of the present invention suggest that this process occurs somewhat differently.

The propensity of the p55-IC to self associate indicates that this domain plays an active role in its induced aggregation. Moreover, this activity of the p55-IC seems to suffice for initiating its signaling, since when expressed independently of the rest of the receptor molecule, it can trigger cell death in the absence of TNF or any other exterior stimuli. Nevertheless, when expressed as the full length receptor, the p55-TNF-R does not signal, unless stimulated by TNF. One must, therefore, assume that when activating the p55-TNF-R, TNF actually overcomes some inhibitory mechanisms, which prevent spontaneous association of the intracellular domains, and this inhibition is due to the linkage of the p55-IC to the rest of the receptor molecule. The inhibition may be due to the orientation imposed on the intracellular domain by the transmembrane and extracellular domain, to association of some other proteins with the receptor or perhaps just due to restriction of the amounts of receptors that are allowed to be placed in the plasma membrane. Of note, this control mechanism should be rather effective, since according to some estimations, the binding of even just one TNF molecule to a cell suffices for the triggering of its death.

Spontaneous signaling, independent of ligand can result in extensive derangement of the process controlled by this receptor. The best known example is the deregulation of growth factor receptors. Mutations due to which they start signaling spontaneously, for example those that cause them to aggregate spontaneously, play an important role in the deregulated growth of tumor cells. TNF effects, when induced in excess, are well known to contribute to the pathology of many diseases. The ability of free intracellular domains (p55ICs) of the p55-TNF-R to signal independently of TNF may contribute to such excessive function. It seems possible, for example, that some of the cytopathic effects of viruses and other pathogens result, not from their direct cytocidal function, but from proteolytic detachment of the intracellular domain of the p55-TNF-R and the resulting TNF-like cytotoxic effect.

To further elucidate the region(s) within p55IC which is responsible for its self-association capability and hence its ligand-independent cell cytotoxicity, and also to determine whether other related members of the TNF/NGF receptor family (e.g. FAS-R) also have intracellular domains with self-association capabilities and ligand-independent effects, the following detailed study was performed :

(a) General Procedures and Materials

(i) Two hybrid screen and two-hybrid β-galactosidase expression test

cDNA inserts, encoding the p55-IC and its deletion mutants, the Fas-IC and various other proteins (see Table 3), were cloned by PCR, either from the full-length cDNAs cloned previously in our laboratory, or from purchased cDNA libraries. β-galactosidase expression in yeasts (SFY526 reporter strain (Bartel et al., 1993)) transformed with these cDNAs in the pGBT-9 and pGAD-GH vectors (DNA binding domain (DBD) and activation domain (AD) constructs, respectively) was assessed by a liquid test (Guarente, 1983); it was also assessed by a filter assay, yielding qualitatively the same results (not shown). Two-hybrid screening (Fields and Song, 1989) of a purchased Gal4 AD-tagged HeLa cell cDNA library (Clontech, Palo Alto, Ca., U.S.A.) for proteins that bind to the intracellular domain of the p55-R (p55-IC), was performed using the *HF7c* yeast reporter strain according to the recommendation of the producer. Positivity of the isolated clones was assessed by (a) prototrophy of the transformed yeasts for histidine when grown in the presence of 5 mM 3-aminotriazole, (b) β-galactosidase expression (c) specificity tests (interaction with SNF4 and lamin fused to Gal4 DBD).

(ii) *In vitro* self-association of bacterially produced p55-IC fusion proteins

Glutathione S-transferase (GST) and glutathione S-transferase-p55-IC fusion protein (GST-p55-IC) were produced as described elsewhere (Frangioni and Neel, 1993; Ausubel et al., 1994). Maltose binding protein (MBP) fusion proteins were obtained using the pMalcRI vector (New England Biolabs) and purified on an amylose resin column. The interaction of the MBPP and GST fusion proteins was investigated by incubating glutathione-agarose beads sequentially with the GST and MBPP fusion proteins (5 μg protein / 20 μl beads; first incubation for 15 min, and the second for 2h, both at 4°C). Incubation with MBP fusion proteins was carried out in a buffer solution containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM dithiotreitol, 0.2% Triton X100, 0.5 mM phenyl-methyl-sulphonyl-fluoride and 5% (v/v) glycerol or, when indicated, in that same buffer containing 0.4 M KCl, or 5 mM EDTA instead of $MgCl_2$. Association of the MBP fusion proteins was assessed by SDS polyacrylamide gel electrophoresis (10% acrylamide) of the proteins associated with the glutathione-agarose beads, followed by Western blotting. The blots were probed with rabbit antiserum against MBP (produced in our laboratory) and with horseradish-peroxidase-linked goat-anti-rabbit immunoglobulin.

(iii) Induced expression in HeLa cells of the p55-R and fragments thereof

HeLa cells expressing the tetracycline-controlled transactivator developed by Gossen and Bujard (the HtTA-1 clone (Gossen and Bujard, 1992)), were grown in Dulbecco's modified Eagle's medium, containing 10% fetal calf serum, 100 u/ml penicillin, 100 μg/ml streptomycin and 0.5 mg/ml neomycin. cDNA inserts encoding the p55-R or parts thereof were introduced into a tetracycline-controlled expression vector (pUHD 10-3, kindly provided by H. Bujard). The cells were transfected with the expression construct (5 μg DNA/6 cm plate) by the calcium phosphate precipitation method (Ausubel et al., 1994). Effects of transient expression of the transfected proteins were assessed at the indicated times after transfection in the presence or

absence of tetracycline (1 μg/ml). Clones of cells stably transfected with the human p55-IC cDNA in the pUHD 10-3 vector were established by transfecting the cDNA to HtTA-1 cells in the presence of tetracycline together with a plasmid conferring resistance to hygromycin, followed by selected for clones resistant hygromycin (200 μg/ml). Expression of the cDNA was obtained by removal of tetracycline which was otherwise maintained constantly in the cell growth medium.

(iv) **Assessment of TNF-like effects, triggered by induced expression of the p55-R and fragments thereof**

Effects of induced expression of the receptor and of TNF on cell viability were assessed by the neutral-red uptake method (Wallach, 1984). Induction of IL-8 gene expression was assessed by Northern analysis. RNA was isolated using TRI REAGENT (Molecular Research Center, Inc.), denatured in formaldehyde/formamide buffer, electrophoresed through an agarose/formaldehyde gel and blotted to a GeneScreen Plus membrane (Du Pont) in 10xSSPE buffer, using standard techniques. Filters were hybridized with an IL-8 cDNA probe (Matsushima et al., 1988), nucleotides 1-392), radiolabeled by the random-prime kit (Boehringer Mannheim Biochemica, Mannheim, Germany) and washed stringently according to the protocol of manufacturer. Autoradiography was performed for 1-2 days.

(v) **Assessment of TNF receptor expression**

TNF receptor expression in samples of $1x10^6$ cells was assessed by measuring the binding of TNF, labeled with ^{125}I by the chloramine-T method, as previously described (Holtmann and Wallach, 1987). It was also assessed by ELISA, performed as described for the quantification of the soluble TNF receptors (Aderka et al., 1991), except for the use of RIPA buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS and 1 mM EDTA) to lyse the cells (70 μl/10^6 cells) and to dilute the tested samples. The soluble form of the p55-R, purified from urine, served as the standard.

(b) Mutational analysis of the intracellular domain of the p55-R (p55-IC) to determine the regions of the p55-IC involved in its self-association

As noted above, p55-IC can self-associate and trigger cytotoxic effects on cells, and there are portions of the p55-IC, which themselves were capable of binding to the full-length p55-IC. In particular, one of the portions of the p55-IC (designated as protein fragment 55.1 in Example 1 above) was identified that was capable of binding strongly to the full length p55-IC, this portion was sequenced and was observed to contain the amino acid residues 328-426 of the p55-TNF-R, which are within the p55-IC. It has further been discovered (see below) that the above portion, protein fragment 55.1, is itself capable of self-association and of triggering cytotoxic effects on cells. Hence this portion of the p55-IC has been called the 'death domain', and is located in the region between amino acid residues 328-426 of the human p55-R, most likely consisting of amino acid residues between about residue 328 and 414 thereof.

The fact that the 'death domain' in the p55-IC self-associates was found by happenstance. On screening a HeLa cell cDNA library by the two-hybrid technique (see Example 1 above) for proteins that bind to the intracellular domain of this receptor, we detected among the cDNAs whose products bound specifically to the intracellular domain-GAL4 DBD fusion-protein, several

clones (e.g. 55.1 and 55.3) that themselves encoded for parts of the p55-R intracellular domain (p55-IC; marked with asterisks in Table 3).

Applying the two-hybrid test to evaluate the extent of specificity in the self-association of p55-IC and to define more accurately the region involved led to the following findings (Table 3) : (a) The self-association of p55-IC is confined to a region within the 'death domain'. Its N terminus is located between residues 328 and 344 and its C terminus, close to residue 404, somewhat upstream of the reported C terminus of this domain (residue 414). (b) Deletion of the membrane-proximal part of p55-IC upstream of the 'death domain' enhanced self-association, suggesting that this region has an inhibitory effect on the association. (c) Mouse p55-IC self-associates, and also associates with the 'death domain' of human p55-R. (d) Examination of the self-association of the intracellular domains of three other receptors of the TNF/NGF receptor family : Fas/APO1 (FAS-R), CD40 (Fields and Song, 1989) and the p75 TNF receptor (Smith et al., 1990), showed that Fas-IC, which signals for cell death by a sequence motif related to the p55-R 'death domain', self-associates, and associates to some extent with the p55-IC. However, CD40-IC, that provides growth stimulatory signals (even though also containing a sequence resembling the 'death domain'), and p75-IC, that bears no structural resemblance to p55-IC, do not self associate, nor do they bind p55-IC or Fas-IC.

TABLE 3. Self-association of the intracellular domains of p55-R and Fas/APO1 within transformed yeasts : assessment by a two-hybrid β-galactosidase expression test.

	HUMAN p55-IC & DELETION MUTANTS								OTHER PROTEINS					
	206-426	278-426	328-426*	328-414	328-404	328-373	344-414	206-326	mouse p55-IC	mouse Fas-IC	human CD40-IC	human p75-IC	SNF4	pGAD-GII
HUMAN p55-IC & DELETION MUTANTS														
206-426 (p55-IC)	5.0	3.6	44.5	30.3	3.5	0	0	0	—	—	—	—	—	0
328-426*	5.2	—	63.5	—	—	—	—	—	—	—	—	—	—	0
367-426	0	—	—	—	—	—	—	—	—	—	—	—	—	—
206-345	0	—	0	—	—	—	—	—	—	—	—	—	—	—
206-326	0	—	0	—	—	—	0	—	—	—	—	—	—	—
OTHER PROTEINS														
mouse p55-IC	0.17	0.38	3.0	—	—	—	—	—	2.5	—	—	—	—	0
mouse Fas-IC	0	0	0.14	—	—	—	—	—	0	38.8	—	0	0	0
hu CD40-IC	0	0	0	—	—	—	—	—	0	0	0	—	—	0
human p75-IC	0	—	—	—	—	—	—	—	—	0	—	0	—	0
SNF1	—	—	—	—	—	—	—	—	—	—	—	—	2.7	0
Lamin	0	0	0	—	—	—	—	—	—	0	—	—	—	0
pGBT9	0	0	0	—	—	—	—	—	0	0	—	—	—	—

Table 3 above shows the quantitative assessment of the interaction of Gal4 hybrid constructs encompassing the following proteins : the intracellular domain of human p55-R and its various deletion mutants (residues numbered as in (Loetscher et al., 1990)); the intracellular domains of mouse p55-R (residues 334-454, numbered as in (Goodwin et al., 1991)); mouse Fas/APO1 (Fas-IC, 166-306, numbered as in (Watanabe-Fukanaga et al., 1992)); human CD40 (CD40-IC, 216-277, numbered as in (Stamenkovic et al., 1989)); and human p75 TNF receptor (p75-IC, 287-461, numbered as in (Smith et al., 1990)). SNF1 and SNF4 were used as positive controls for association (Fields and Song, 1989), and lamin as a negative control (Bartel et al., 1993). Proteins encoded by the Gal4 DBD constructs (pGPT9) are listed vertically; those encoded by the Gal4 AD constructs (pGAD-GH), horizontally. The two deletion mutants denoted by asterisks were cloned in a two-hybrid screen of a HeLa cell cDNA library (Clontech, Palo Alto, Ca., U.S.A.) using p55-IC cloned in pGBT9 as "bait". In that screen, four of about 4×10^6 cDNA clones examined were positive. Three of these clones were found to correspond to parts of human p55-R cDNA (two were identical, encoding residues 328–426 and one encoding residues 277-426). The fourth was found to encode an unknown protein. The β-galactosidase expression data are averages of assays of two independent transformants and are presented as amount of β-galactosidase product; (a unit of activity being defined as OD_{420} times 10^3 divided by OD_{600} of the yeast culture and reaction time, in minutes). The detection limit of the assay was 0.05 units. Variation between duplicate samples were in all cases less that 25% of the average (not tested).

An *in vitro* test of the interaction of a p55-IC-glutathione-S-transferase (GST) bacterial fusion protein with a p55-IC-maltose binding protein (MBP) fusion protein confirmed that p55-R self-associates and ruled out involvement of yeast proteins in this association (see above). The association was not affected by increased salt concentration, nor by EDTA (see above).

To evaluate the functional implications of the self-association of the death domain, we examined the way in which induced expression of p55-R, or of parts of it, affect cells sensitive to TNF cytotoxicity. The results of this analysis are set forth in Fig. 7 which depicts the ligand-independent triggering of a cytocidal effect in HeLa cells transfected with p55-R, its intracellular domain (p55-IC) or parts thereof (including the 'death domain').

In Fig. 7 there is shown schematically, the various DNA molecules encoding the different types of TNF receptors included in the vectors with which the HeLa cells were transfected (extreme left hand side of Fig. 7); and the expression (left and middle bar graphs) and the viability (right bar graph) in HeLa cells expressing transiently the various full-length p55-R (p55-R), p55-IC or parts of p55-IC or, as a control, luciferase (LUC) (each being depicted at the extreme left side of Fig. 7), using a tetracycline-controlled expression vector. The open bar graphs (left, middle and right) represent cells transfected in the presence of tetracycline (1 μg/ml), which inhibits expression; and the filled bar graphs (left, middle and right) represent cells transfected in the absence of tetracycline. TNF receptor expression was assessed 20h after transfection, both by ELISA, using antibodies against the receptor's extracellular domain (see schematic illustration on the left side of Fig. 7), and by determining the binding of radiolabeled TNF to the cells (middle). The cytocidal effect of the transfected proteins was assessed 48h after transfection. Data shown

are from one of three experiments with qualitatively similar results, in which each construct was tested in duplicate. ND - not determined.

Thus, from Fig. 7 it is apparent that by using an expression vector that permits strictly controlled expression of transfected cDNAs by a tetracycline regulated transactivator (Gossen and Bujard, 1992), a mere increase of p55-R expression in HeLa cells by expression of transiently transfected cDNA for the full-length receptor resulted in quite extensive cell death. An even greater cytotoxicity was observed when expressing just p55-IC. Significant cytotoxicity was also observed when expressing just a part of p55-IC comprising essentially the 'death domain' (residues 328-426) in the HeLa cells. On the other hand, expression of parts of p55-IC that lacked the 'death domain' or contained just part of it (or expression of the luciferase gene, used as an irrelevant control) had no effect on cell viability. The cytotoxicity of p55-IC was further confirmed using cells stably transformed with its cDNA; these cells continued to grow when p55-IC expression was not induced, but died when p55-IC was expressed (see above).

(c) Other effects of the intracellular domain of the p55-TNF-R

To examine whether other activities of TNF are triggered by the self-association of the intracellular domain, including the 'death domain' thereof, we examined the effect of increased expression of the full-length receptor (p55-R) and of the expression of the intracellular domain of the receptor (p55-IC), on the transcription of interleukin 8 (IL-8), known to be activated by TNF (Matsushima et al., 1988). The results are shown in Fig. 8, which depicts the ligand-independent induction of IL-8 gene expression in HeLa cells transfected with p55-R or p55-IC, using a tetracycline-controlled construct (see also 'General Procedures and Materials' and Example 1 above). In panel A of Fig. 8 there is shown a reproduction of a Northern blot representing the Northern blot analysis (see 'General Procedures and Materials' above) of RNA (7 μg/lane) extracted from HeLa (HTta-1) cells, untreated ('control') or treated ('TNF') with TNF (500 μ/ml for 4h), or the HTta-1 cells 24h after transfection (in the presence or absence of tetracycline) with p55-IC ('p55-IC'), the p55-R ('p55-R'), or luciferase ('Luc') cDNA. In panel B of Fig. 8 there is shown a reproduction of a Northern blot representing the methylene blue staining of 18S rRNA in each of the samples shown in panel A of Fig. 8.

Thus, as is apparent from Fig. 8, transfection of HeLa cells with a tetracycline-controlled construct encoding the p55-R cDNA induced IL-8 transcription. An even stronger induction was observed in cells transfected with the cDNA for p55-IC. In both cases, the induction occurred only when tetracycline was excluded from the cell growth medium, indicating that it occurs as a consequence of expression of the transfected p55-R or p55-IC. Transfection with luciferase cDNA, as a control, had no effect on IL-8 transcription.

Accordingly, from the above results (Fig. 8), it appears that a mere increase in p55-R expression, or even expression of just the intracellular domain (p55-IC) thereof is sufficient to trigger, in a ligand (TNF)-independent fashion, cytotoxicity and other effects as well, including that of an increase in the expression of the IL-8 gene within cells. The triggering of these effects is most likely due to the self-association of the intracellular domain of the p55-R (p55-IC). As is set forth above, it appears that, upon self-association of the p55-IC, the 'death domain' th reof is

primarily responsible for signaling the induction of the intracellular processes leading to the triggering of cytotoxicity within the cells, whilst the other effects, e.g the signaling leading to the induction of IL-8 gene expression, are likely due to other regions of the p55-IC as well, following the self-association thereof. It is therefore possible that different regions of the p55-IC are responsible for the different TNF-induced effects (e.g. cytotoxicity, IL-8 induction) within cells, these effects being a consequence of the intracellular signaling upon self-association of the p55-IC.

The fact that the p55-IC, can induce in a ligand (TNF)-independent fashion, the triggering of other intracellular effects e.g. IL-8 induction, means that the p55-IC or specific portions thereof may be used as a highly specific tool for bringing about such effects in cells or tissues that it is desired to treat, without the need for treating such cells or tissues with TNF. In many pathological conditions (e.g. malignancies), treatment with TNF, especially at high dosages can lead to undesirable side-effects due to the number of intracellular effects induced systemically by TNF following its binding to its receptors. By way of the discovery in accordance with the present invention that the p55-IC can mimic specific other TNF-induced effects (besides cytotoxicity), e.g. IL-8 induction, opens the way for introducing in a cell- or tissue-specific manner, p55-IC or specific portions thereof, which will be capable of signaling for the induction of specific desired intracellular effects, e.g. IL-8 induction, and thereby overcome the systemic side-effects often observed during TNF treatment.

(d) Ligand-independent triggering of cytocidal effects in HeLa cells by the intracellular domains and the 'death domains' thereof of p55 TNF-R and FAS-R (Fas/APO1)

As regards the cytotoxic activity of the intracellular domains of the p55 TNF-R and FAS-R (p55IC and FAS-IC) it has now also been further elucidated that both the p55IC, its 'death domain' (p55DD) and the FAS-IC are capable of a ligand-independent triggering of a cytocidal effect in HeLa cells. In this study, HeLa cells were transfected with expression vectors containing various constructs of either the full-length p55-TNF-R, portions thereof including the p55IC and p55DD or the FAS-IC. In one set of experiments HeLa cells were co-transfected with constructs containing the p55 TNF-R (p55-R) and the FAS-IC (for details of the constructs, their preparation, etc. see above). The results of this study are depicted in Fig. 9 (A and B), wherein in both Fig. 9A and B the constructs used for transfecting the HeLa cells are shown schematically in the left hand panels; the results of the TNF or FAS receptor expression are shown graphically in the two middle panels (second and third panels from the left), and the results of transfected cell viability are shown graphically in the right hand panels. In Fig. 9A there is shown the results of transfected HeLa cells transiently expressing the full-length p55-R, p55-IC or parts thereof, or as a control, luciferase (LUC), in all cases using a tetracycline-controlled expression vector. In Fig. 9B there is shown the results of transfected HeLa cells transiently expressing FAS-IC alone or together with the p55-R, using a tetracycline-controlled expression vector. In the graphic representation of the results in Fig. 9A and B, the open bars represent cells transfected in the presence of tetracycline (1 µg/ml), which inhibits expression, and the closed bars represent cells transfected in the absence of tetracycline. TNF receptor expression was assessed 20h after

transfection, both by ELISA using antibodies against the extracellular domain of the receptor (see left hand panels), and by determining the binding of radiolabeled TNF to the cells (middle panels). The cytocidal effect of the transfected proteins was assessed 48h after transfection. The data shown are from one of three experiments with qualitatively similar results in which each construct was tested in duplicate. The designation 'ND' in Figs. 9A and B means not determined. From the results shown in Figs. 9A and B it is apparent that expression of only the p55IC results in even greater cytotoxicity. Significant cytotoxicity also occurs when expressing just the death domain (p55DD). In contrast, expression of parts of p55IC lacking the death domain or containing only part thereof, had no effect on cell viability. Expression of the FAS-IC did not result in significant cytotoxicity, yet it significantly enhanced the cytotoxicity of co-expressed p55-R.

EXAMPLE 3 :

Additional proteins capable of binding to the intracellular domains of p55 TNF-R or FAS-R

Using the same approach and technology set forth in Example 1 above, three more proteins have been isolated and identified which are capable of binding to the p55IC or FAS-IC.

In Figs. 10-12 there is shown schematically the partial and preliminary nucleotide sequence of cDNA clones, called F2, F9 and DD11, respectively.

Clones F2 and F9 were isolated by screening a murine (mouse) embryonic library using the murine FAS-IC as "bait". In Fig. 10 there is shown schematically the partial nucleotide sequence from the F2 cDNA that has been sequenced. In Fig. 11 there is shown schematically the partial nucleotide sequence of 1724 bases from the F9 cDNA that has been sequenced. Analysis of the binding capability of the protein encoded by clones F2 and F9 (F2 and F9, respectively) has shown that :

(a) F2 interacts strongly with human p55IC and p55DD and with murine FAS-IC, while it interacts weakly with non-relevant (control) proteins SNF1 and Lamin as well as relevant protein, human FAS-IC.

(b) F9 interacts strongly with human p55-IC and murine FAS-IC, while it interacts weakly with human FAS-IC (relevant protein) and irrelevant proteins SNF1 and Lamin.

(c) Neither F2 nor F9 interacted at all with human p75IC, pGBT9 (empty bait vector), or human CD-40.

Further, from 'Gene Bank' and 'Protein Bank' searches it was revealed that F2 and F9 represent new proteins.

Thus, F2 and F9 represent new proteins having binding specificity for both FAS-IC and p55IC.

Clone DD11 was isolated by screening a human HeLa library using the human p55DD as "bait". In Fig. 12 there is shown schematically the partial nucleotide sequence of 425 bases from the DD11 cDNA that has been sequenced.

The DD11 clone has an approx. length of 800 nucleotides. The full length of the transcript is about 1.2 kb, the transcript having been probed using the clone. Analysis of the binding

capability of the protein encoded by clone DD11 has shown that DD11 interacts strongly with the p55DD (a.a. 326-414) (see Fig. 9) and does not interact with deletion mutants of this domain, e.g. a.a. 326-404. DD11 also interacts with mouse and human FAS-IC and to some extent also with Lamin. DD11 does not interact at all with SNF1 nor with pGBT9 (empty bait vector). DD11 is also not found in the 'Gene Bank' and 'Protein Bank' databases. Thus DD11 represents a p55 IC (p55DD) and FAS-IC specific binding protein.

Example 4

Construction of soluble dimeric TNF receptors

Based on the findings set forth in Example 2 above, that the intracellular domain of the p55-R (p55-IC) and a portion thereof (the 'death domain'), and that the intracellular domain of the Fas/APO1 and a portion thereof (also called the 'death domain') which resembles the p55-IC 'death domain', are capable of self-association, it is possible to construct new TNF receptors which are capable of self-association (aggregation) and which are soluble. Such TNF receptors will be fusion proteins having essentially all of the extracellular domain of the p55-R fused to essentially all of the intracellular domains or 'death domains' thereof of the p55-R or Fas/APO1. Thus, such fusion constructs will be devoid of the transmembranal domain of the p55-R (or FAS/APO1) and hence will be soluble. Moreover, by virtue of the self-association capability of the intracellular domains or 'death domains' thereof, these fusion constructs will be capable of oligomerization to provide at least dimers (and possibly also higher order multimers) of the p55-R. Consequently, such dimeric TNF receptors (p55-R) will be capable of binding to at least two TNF monomers of the naturally-occurring TNF homotrimer to provide a soluble TNF receptor which binds more avidly to its ligand (homotrimeric TNF).

Accordingly, at least four types of p55 TNF receptor fusion proteins will be constructed each of which will be capable of oligomerization and will be soluble :

(i) A fusion product between the extracellular domain of p55-R (EC55) and the intracellular domain of p55-R (p55-IC);

(ii) A fusion product between the EC55 and the 'death domain' of p55-IC (DD55);

(iii) A fusion product between the EC55 and the intracellular domain of Fas/APO1 (ICFAS); and

(iv) A fusion product between the EC55 and the 'death domain' of ICFAS (DDFAS).

In each of the above fusion proteins the TNF monomer binding capability is provided by the EC55 portion while the oligomerization (or at least dimerization) of each kind of fusion protein is provided by its 'tail' region being any of the p55IC, DD55, ICFAS or DDFAS portions.

For construction of the above fusion proteins, standard techniques of recombinant DNA technology will be employed that are now well established in the art (see for example Sambrook et al., (1989) Molecular Cloning : A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Briefly, any suitable bacterial, bacteriophage, or animal virus expression vector (cloning vehicle or plasmid designed for expression of the inserted DNA of

choice) may be employed into which will be inserted in one or more stages the DNA encoding the EC55 and one of the 'tails' being the p55-IC, DD55, ICFAS or DDFAS. The so-inserted DNA encoding each of the fusion proteins will be placed under the control of the various expression control sequences of the cloning vehicle or plasmid such as promoters, ribozyme binding sites, transcriptional factor binding sites, etc. These expression control sequences will be chosen depending on the type of expression vector chosen and hence the type of host cell (eukaryotic or prokaryotic) in which it is desired to express the fusion proteins of the invention. Preferred host cells (and hence expression vectors) are eukaryotic, in particular, mammalian.

The DNA molecule encoding each of the above noted fusion proteins will be prepared and inserted into the expression vector by the following procedure :

(a) Firstly, a set of oligonucleotides for use in PCR will be constructed by standard means, the oligonucleotides being :

1) ACC ATG GGC CTC TCC ACC GTG (EC55, sense)
2) ACGC GTC GAC TGT GGT GCC TGA GTC CTC (EC55, antisense)
3) ACGC GTC GAC CGC TAC CAA CGG TGG AAG (IC55, sense)
4) TCA TCT GAG AAG ACT GGG (IC55, antisense
5) ACGC GTC GAC AAG AGA AAG GAA GTA CAG (IC FAS, sense)
6) CTA GAC CAA GCT TTG GAT (IC FAS, antisense)
7) ACGC GTC GAC CCC GCG ACG CTG TAC GCC (DD55, sense)
8) ACGC GTC GAC GAT GTT GAC TTG AGT AAA (DD FAS, sense)

(b) Plasmids containing the cloned full-length p55-R and Fas/APO1 receptors which we have in our laboratory (see also co-pending EP568925 and Examples 1-3 above) will be subjected to the following manipulations to yield the DNA fragments encoding each of the fusion proteins, which DNA fragments are then ligated into the above noted expression vector of choice :

(i) To produce the DNA fragment coding for EC55 which is a component of all 4 fusion proteins, PCR is performed on a plasmid bearing cDNA of human p55 using the above oligonucleotide nos. 1 and 2 (size of fragment 640 bp).

(ii) To get a fusion product EC55-IC55, PCR is performed on a plasmid bearing cDNA for human p55 using oligonucleotide nos. 3 and 4, to obtain a DNA fragment coding for IC55 (size 677 bp) which is then mixed with EC55 digested by Sal I and ligated by blunt end ligation into any expression vector for mammalian cells under the control of an appropriate promoter. The orientation of the inserted EC55--IC55 in the vector is verified by restriction digestion and by sequencing.

(iii) To get a fusion product EC55–IC FAS, IC FAS is produced by PCR on a plasmid with cDNA for FAS using oligonucleotide nos. 5 and 6, to obtain a fragment (size 448 bp) which is then cut by Sal I and mixed with EC55 cut by SalI, and subsequently is blunt ligated into a mammalian expression vector under the control of an appropriate promoter. The orientation of the inserted EC55--IC FAS in the vector is verified by restriction digestion and by sequencing.

(iv) To get a fusion product EC55--DD55, a DNA fragment is produced with the DD55 sequence by PCR in cDNA for human p55 using oligonucleotide nos. 7 and 4. The product with a size of 314 bp is cut by SalI and mixed with EC55 cut by SalI, and subsequently blunt ligated into the mammalian expression vector. Orientation of the inserted EC55--DD55 in the vector is verified by restriction digestion and by sequencing.

(v) To get a fusion product EC55--DD FAS, a DNA fragment with DD FAS is produced by PCR on cDNA for FAS using oligonucleotide nos. 6 and 8. The product with a size of 332 bp is cut with SalI, and mixed with EC55 cut by Sal I and subsequently blunt ligated into the mammalian expression vector. Orientation of the EC55--DD FAS is then verified by restriction digestion and sequencing

Once the above expression vectors have been constructed, they will then be introduced by standard methods into suitable mammalian cells (e.g. Chinese Hamster Ovary (CHO) or Monkey Kidney (COS) cells) for the purposes of expression. The so-expressed fusion proteins will then be purified by standard methods (see co-pending EP308378; EP398327; and EP568925). The purified fusion proteins will then be analyzed for their ability to oligomerize (and the extent thereof, i.e. whether they form dimers or higher order multimers) and for their ability to bind TNF (and the affinity or avidity of binding thereof).

Example 5

Construction of soluble dimeric Fas/APO1 receptors

In a similar fashion to that set forth in Example 4 above, it is possible to produce the following four kinds of Fas/APO1 fusion products, each of which will be capable of oligomerization and will be soluble :

(i) Fusion product between the extracellular domain of Fas/APO1 (EC FAS) and the intracellular domain of p55-IC;

(ii) Fusion product between the EC FAS and the 'death domain' of p55-IC (DD55);

(iii) Fusion product between the EC FAS and the intracellular domain of Fas/APO1 (IC FAS); and

(iv) Fusion product between the EC FAS and the 'death domain' of IC FAS (DD FAS).

In each of the above fusion proteins the FAS ligand binding capability is provided by the EC FAS portion, while the oligomerization (or at least dimerization) of each kind of fusion protein is provided by its 'tail' region being any of the p55-IC, DD55, IC FAS or DD FAS portions.

The construction of the DNA fragments encoding the above fusion proteins and expression vectors containing them will be as detailed in Example 4, except different appropriate oligonucleotides (not shown) will be used for the preparation of the EC FAS fragment to be ligated to any of the above noted 'tail' regions. Subsequently, the expression vectors will be introduced into the suitable host cells, and the resulting expressed fusion proteins will be purified

and tested for their ability to oligomerize (and the extent thereof, i.e. whether they form dimers or higher order multimers) and for their ability to bind the FAS ligand (and the affinity or avidity of binding thereof).

Example 6

Construction of soluble oligomeric 'mixed' TNF/FAS receptors

To prepare oligomeric receptors having 'mixed' affinity, i.e. affinity for both TNF and the FAS-R ligand, the above-mentioned (Examples 4 and 5) fusion products may be utilized in the following procedure :

i) Providing a fusion product as set forth in Example 4, which contains the extracellular domain of a TNF-R (p75 TNF-R or p55 TNF-R) fused to any one of : the p55 IC, FAS-IC, p55 DD or FAS DD;

ii) Providing a fusion product as set forth in Example 5, which contains the extracellular domain of Fas-R fused to any one of : p55 IC, FAS-IC, p55 DD or FAS-DD; and

iii) mixing any one of the fusion products of i) with any one of the fusion products of ii) to provide a new dimeric (or higher order oligomeric) receptor which has both the extracellular domains of a TNF-R and FAS-R that are joined by their -IC or -DD regions.

In the above procedure the fusion products of i) and ii) may be provided separately, namely, from their purification from transformed cells in which they were produced, and then mixed *in vitro* to obtain the mixed affinity receptors. Alternatively, the host cells may be co-transfected with vectors carrying sequences encoding both types of fusion products, in which case, the mixed affinity receptors may be obtained directly from the co-transfected cells. The actual oligomerization of the fusion products into oligomeric receptors may take place within the cells or during or following the purification procedure to obtain the fusion products expressed in the cells. To specifically select for the mixed affinity receptors any standard method may be utilized, for example, affinity chromatography procedures in which antibodies against the TNF-R and FAS-R extracellular domains are used in sequential chromatographic steps to select for those receptors having both types of extracellular domain.

REFERENCES

Aderka, D., Englemann, H., Hornik, V., Skornick, Y., Levo, Y., Wallach, D. and Kushtai, G. (1991) Cancer Res. 51, 5602-5607.

Baens et al. (1993) Genomics 16:214-218.

Barinaga, M. (1993) Science 2621512-4.

Bartel, P.L., Chien, C.T., Sternglanz, R. and Fields, S. (1993) Bio Techniques 14, 920-924.

Berger, J., Hauber, J., Hauber, R., Geiger, R. and Cullen, B.R. (1988) Gene 66, 1-10.

Beutler, B. and Cerami, C. (1987) NEJM, 316:379-385.

Boldin, M.P. et al. (1995) J. Biol. Chem. 270, 337-341.

Bollon, D.P. et al. (1980) J. Clin. Hematol. Oncol. 10, 39-48.

Botstein, D. et al. (1982) Miami Wint. Smyp. 19, 265-274.

Brakebusch, C. et al. (1992) EMBO J., 11:943-950.

Broach, J.R. (1981) in : The Molecular Biology of the Yeast Saccharomyces : Life Cycle and Inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 445-470.

Broach, J.R. (1982) Cell 28, 203-204.

Brockhaus, M. et al. (1990) Proc. Natl. Acad. Sci. USA, 87:3127-3131.

Cantor, G.H. et al. (1993) Proc. Natl. Acad. Sci. USA 90:10932-6.

Chater, K.F. et al. (1986) in : Sixth International Symposium on Actinomycetales Biology, Akademiai Kaido, Budapest, Hungary, 45-54.

Chen, C.J. et al. (1992) Ann N.Y. Acad. Sci. 660:271-3.

Cheng, J., Zhou, T., Liu, C. Shapiro, J.P. Brauer, M., Kiefer, M.C., Barr, P.J. and Mountz, J.D. (1994) Science 263, 1759-1762.

Crisell, P. et al., (1993) Nucleic Acids Res. (England) 21 (22):5251-5.

Crowe, P.D. et al., (1994) Science, 264:707-709.

Current protocols in molecular biology (Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., Albright, L.M., Coen, D.M. & Varki, A., eds.), (1994) pp. 8.1.1-8.1.6 and 16.7-16.7.8, Greene Publishing Associates, Inc. and Wiley & Sons, Inc., New York.

DeMartino, G.N., Moomaw, C.R., Zagnitko, O.P., Proske, R.J., Chu-Ping, M., Afendis, S.J., Swaffield, J.C. and Slaughter, C.A. (1994) J. Biol. Chem. 269, 20878-20884.

Dirks, W., Wirth, M. and Hauser, H. (1993) Gene 128, 247-249.

Endo, H., Akahoshi, T., Nishimura, A., Tonegawa, M., Takagishi, K., Kashiwazaki, Matsushima, K. and Kondo, H. (1994) Clin. Exp. Immunol. 96, 31-35.

Engelmann, H. et al. (1990) J. Biol. Chem., 265:1531-1536.

Ferrick, M.R., Thurau, S.R., Oppenheim, M.H., Herbort, C.P., Ni, M., Zachariae, C.P., Matsushima, K. and Chan, C.C. (1991) Invest. Ophthalmol. Vis. Sci. 32, 1534-1539.

Fields, S. and Song, O. (1989) Nature, 340:245-246.

Frangioni, J.V. and Neel, B.G. (1993) Anal. Biochem. 210, 179-187.

Glick, B.R. (1987) J. Ind. Microbiol. 1, 277-282.

Goodwin, R.G., Anderson, D., Jerzy, R., David, T., Brannan, C.I., Copeland, N.G., Jenkins, N.A. and Smith, C.A. (1991) Mol. Cell Biol. 11, 3020-3026.

Gossen, M. and Boujard, H. (1992) Proc. Natl. Acad. Sci. USA, 89:5547-5551.

Gryczan, T. (1982) The Molecular Biology of the Bacilli, Academic Press, N.Y. 307-329.

Guarente, L. (1983) in Methods Enzymol. 101, 181-191.

Harada, A., Sekido, N., Kuno, A., Akiyama, M., Kasahara, T., Nakanishi, I., Mukaid, and Matsushima, K. (1993) Int. Immunol. 5, 681-690.

Heller, R.A. et al. (1990) Proc. Natl. Acad. Sci. USA, 87:6151-6155.

Hohmann, H.-P. et al. (1989) J. Biol. Chem., 264:14927-14934.

Holtmann, H. and Wallach, D. (1987) J. Immunol. 139, 1161-1167.

Itoh, N. et al. (1991) Cell 66:233.

Itoh, N. and Nagata, S. (1993) J. Biol. Chem. 268, 10932-7.

Izaki, K. (1978) Jpn. J. Bacteriol. 33, 729-742.

John, J.F. et al. (1986) Rev. Infect. Dis. 8, 693-704.

Joseph, S. and Burke, J.M. (1993) J. Biol. Chem. 268:24515-8.

Kendall, K.J. et al (1987) J. Bacteriol 169, 4177-4183.

Khan, A.S. et al. (1992) Nature *Genetics*, 2: 180-185.

Koizumi, M. et al. (1993) Biol. Pharm. Bull (Japan) 16 (9):879-83.

Kunkel, T.A. (1994) in : Current protocols in molecular biology, pp. 8.1.1-8.1.6 (Ausubel, F.M. et al., eds.) Greene Publishing Associates, Inc. and Wiley & Sons, Inc., New York.

Loetscher, H., Pan, Y-C.E., Lahm, H.-W, Gentz, R., Brockhaud, M., Tabuchi, H. and Lesslauer, W. (1990) Cell, 61:351-359.

Maniatis, T. et al. (1982) Molecular Cloning : A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor.

Maniatis, T. (1980) in : Cell Biology : A Comprehensive Treatise Vol. 3 : Gene Expression, Academic Press, N.Y. 563-608.

Matsushima, K., Morishita, K., Yoshimura, T., Lavu, S., Kobayashi, Y., Lew, W., Appella, E., Kung, H.F., Leonard, E.J. and Oppenheim, J.J. (1988) J. Exp. Med. 167, 1883-1893.

Nophar, Y. et al. (1990) EMBO J., 9:3269-3278.

Oehm, A. et al. (1992) J. Biol. Chem. 267:10709.

Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitamura, Y., Itoh, N., Suda, T. and Nagata, S. (1993) Nature 364, 806-809.

Okayama, H. (1983) Mol. Cell Biol. 3, 280.

O'Neal, K.D. and Yu-Lee, L.Y. (1993) Lymphokine Cytokine Res. 12, 309-312.

Piquet, P.F. t al. (1987) J. Exp. Med., 166:1280-89.

Realini, C., Rogers, S.W. and Rechsteiner, M. (1994) FEBS Lett 348, 109-113.

Rechsteiner, M., Hoffman, L. and Dubiel, W. (1993) J. Biol. Chem. 268, 6065-6068.

Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring HarborLaboratory Press, Cold spring Harbor, NY.

Schall, T.J. et al. (1990) Cell, 61:361-370.

Schwalb et al. (1993) J. Biol. Chem. 268 (14) :9949-54.

Seger, R. and Krebs, E.G. (1995) FASEB J. in press.

Sekido, N., Mujaida, N., Harada, A., Nakanishi, I., Watanabe, Y., Matsushima, K. (1993) Nature 365, 654-657.

Shimayama, T. et al., (1993) Nucleic Acids Symp. Ser. 29:177-8

Shore, S.K. et al. (1993) Oncogene 8:3183-8.

Smith, C.A., David, T., Anderson, D., Solam, L., Beckmann, M.P., Jerzy, R., Dower, S.K., Cosman, D. and Goodwin, R.G. (1990) Science, 248:1019-1023.

Smith, D.B. and Corcoran, L.M. (1994) in : Current protocols in molecular biology, pp. 16.7.1-16.7.8 (Ausubel, F.M. et al., eds.) Greene Publishing Associates, Inc. and Wiley & Sons, Inc. New York.

Song, H.Y. et al. (1994) J. Biol. Chem. 269, 22492-22495.

Stamenkovic, I., Clark, E.A. and Seed, B. (1989) Embo J. 8:1403-1410.

Tartaglia, L. A., Ayres, T.M., Wong, G.H. and Goeddel, D.V. (1993) Cell, 74:845-853.

Tracey, J.T. et al. (1987) Nature, 330:662-664.

Wallach, D. (1984) J. Immunol. 132, 2464-9.

Wallach, D. (1986) in : Interferon 7 (Ion Gresser, ed.), pp. 83-122, Academic Press, London

Wallach, D. et al. (1994) Cytokine 6, 556.

Watanabe-Fukanaga, R., Brannan, C.I., Itoh, N., Yonehara, S., Copeland, N.G., Jenkins, N.A. and Nagata, S. (1992) J. Immunol. 148, 1274-1279.

Watanabe-Fukunaga, R. et al. (1992) Nature, 356, 314-317.

Wiegmann, K., Schutze, S., Machleidt, T., Witte, D. and Kronke, M. (1994) Cell 78, 1005-1015.

Wilks, A.F. et al. (1989) Proc. Natl. Acad. Sci. USA, 86:1603-1607.

Zhao, J.J. and Pick, L. (1993) Nature (England) 365:448-51.

CLAIMS

1. A DNA sequence encoding a protein capable of binding to one or more of the intracellular domains of one or more receptors belonging to the tumor necrosis factor/nerve growth factor (TNF/NGF) receptor superfamily.

2. A DNA sequence according to claim 1, wherein said receptors are the TNF-Rs, p55 TNF-R or p75 TNF-R, or the FAS ligand receptor (FAS-R).

3. A DNA sequence according to claim 1 or claim 2, selected from the group consisting of:
(a) a cDNA sequence derived from the coding region of a native TNF-R or FAS-R intracellular domain-binding protein;
(b) DNA sequences capable of hybridization to a sequence of (a) under moderately stringent conditions and which encode a biologically active TNF-R or FAS-R intracellular domain-binding protein; and
(c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b) and which encode a biologically active TNF-R or FAS-R intracellular domain-binding protein.

4. A DNA sequence according to any one of claims 1-3, encoding a p55 TNF-R intracellular domain (p55IC)-binding protein.

5. A DNA sequence according to claim 4 encoding a protein selected from the group comprising the herein designated proteins 55.1, 55.3, 55.11, F2, F9 and DD11.

6. A DNA sequence according to claim 5, selected from the sequences contained in the herein designated cDNA clones 55.1, 55.3, 55.11, F2, F9 and DD11.

7. A DNA sequence according to any one of claims 1-3, encoding a p75 TNF-R intracellular domain (p75IC)-binding protein.

8. A DNA sequence according to claim 7, encoding a protein selected from the group comprising the herein designated proteins 75.3 and 75.16.

9. A DNA sequence according to claim 8, selected from the sequences contained in the herein designated cDNA clones 75.3 and 75.16.

10. A DNA sequence according to claim 5 or claim 6 encoding the protein 55.1 having the amino acid sequence from amino acid residue 328 to residue 426 of the p55 TNF-R amino acid sequence.

11. A DNA sequence according to claim 5 of claim 6 encoding the protein 55.3 having the amino acid sequence from amino acid residue 277 to residue 426 of the p55 TNF-R amino acid sequence.

12. A DNA sequence according to claim 5 or claim 6, encoding the protein 55.11 comprising the sequence depicted in Fig. 1(a)

13. A DNA sequence according to claim 8 or claim 9 encoding the protein 75.3 comprising the sequence depicted in Fig. 1(b).

14. A DNA sequence according to claim 8 or claim 9 encoding the protein 75.16 comprising the sequence depicted in Fig. 1(c).

15. A DNA sequence according to any one of claims 1-3 encoding a FAS-R intracellular domain (FAS-IC)-binding protein.

16. A DNA sequence according to claim 15 encoding a protein selected from the group comprising the herein designated proteins F2, F9 and DD11.

17. A DNA sequence according to claim 16 selected from the sequences contained in the herein designated cDNA clones F2, F9 and DD11.

18. A DNA sequence according to claim 16 or 17 encoding any one of the proteins F2, F9 and DD11, comprising the sequences depicted in any one of Figs. 10-12, respectively.

19. A protein or analogs and derivatives thereof encoded by a sequence according to any one of claims 1-18, said protein, analogs and derivatives being capable of binding to one or more of the intracellular domains of one or more TNF-Rs or FAS-R.

20. A protein according to claim 19 selected from the group comprising the proteins 55.1, 55.3. 55.11, 75.3, 75.16, F2, F9 and DD11, and biologically active analogs and derivatives thereof.

21. The protein 55.11 according to claim 20, having the deduced amino acid sequence depicted in Fig. 1(d).

22. The protein 55.11 according to claim 21, further characterized by being capable of binding to the intracellular domain of the p55-TNF-R (p55IC) in the region between residues 243-308 of p55-TNF-R.

23. A vector comprising a DNA sequence according to any one of claims 1-18.

24. A vector according to claim 23 which is capable of being expressed in a eukaryotic host cell.

25. A vector according to claim 23 which is capable of being expressed in a prokaryotic host cell.

26. Transformed eukaryotic or prokaryotic host cells containing a vector according to any one of claims 23-25.

27. A method for producing the protein, analogs or derivatives according to claim 19 or 20 comprising growing the transformed host cells according to claim 26 under conditions suitable for the expression of said protein, analogs or derivatives, effecting post-translational modifications of said protein as necessary for obtention of said protein and extracting said expressed protein, analogs or derivatives from the culture medium of said transformed cells or from cell extracts of said transformed cells.

28. Antibodies or active fragments or derivatives thereof, specific for the protein, analogs or derivatives according to claim 19 or claim 20.

29. A method for the modulation of the TNF or FAS-R ligand effect on cells carrying a TNF-R or a FAS-R, comprising treating said cells with one or more proteins, analogs or derivatives selected from the group consisting of the proteins, analogs and derivatives according to claim 19 or claim 20 and a protein being the p55IC, p55DD, FAS-IC or FAS-DD, analogs or derivatives thereof, all of said proteins being capable of binding to the intracellular domain and modulating the activity of said TNF-R or FAS-R, wherein said treating of said cells comprises introducing into said cells said one or more proteins, analogs or derivatives in a form suitable for intracellular introduction thereof, or introducing into said cells a DNA sequence encoding said one or more proteins, analogs or derivatives in the form of a suitable vector carrying said sequence, said vector being capable of effecting the insertion of said sequence into said cells in a way that said sequence is expressed in said cells.

30. A method according to claim 29 wherein said treating of said cells is by transfection of said cells with a recombinant animal virus vector comprising the steps of:

(a) constructing a recombinant animal virus vector carrying a sequence encoding a viral surface protein (ligand) that is capable of binding to a specific cell surface receptor on the surface of a TNF-R- or FAS-R- carrying cell and a second sequence encoding a protein selected from the proteins, analogs and derivatives according to claim 19 or 20 and a protein being the p55IC, p55DD, FAS-IC or FAS-DD, analogs or derivatives thereof, said protein when expressed in said cells being capable of modulating the activity of said TNF-R or FAS-R; and

(b) infecting said cells with said vector of (a).

31. A method for modulating the TNF or FAS-R ligand effect on cells carrying a TNF-R or a FAS-R comprising treating said cells with antibodies or active fragments or derivatives thereof, according to claim 28, said treating being by application of a suitable composition containing said antibodies, active fragments or derivatives thereof to said cells, wherein when the IC-binding proteins of said cells are exposed on the extracellular surface, said composition is formulated for extracellular application, and when said IC-binding proteins are intracellular said composition is formulated for intracellular application.

32. A method for modulating the TNF or FAS-R ligand effect on cells carrying a TNF-R or FAS-R comprising treating said cells with an oligonucleotide sequence selected from a sequence encoding an antisense sequence of at least part of the sequence according to any one of claims 1-18, and a sequence encoding the antisense sequence of p55IC, p55DD, FAS-IC or FAS-DD, said oligonucleotide sequence being capable of blocking the expression of at least one of the TNF-R or FAS-R intracellular domain binding proteins.

33. A method according to claim 32 wherein said oligonucleotide sequence is introduced to said cells via a virus of claim 30 wherein said second sequence of said virus encodes said oligonucleotide sequence.

34. A method for treating tumor cells or HIV-infected cells or other diseased cells, comprising:

(a) constructing a recombinant animal virus vector carrying a sequence encoding a viral surface protein that is capable of binding to a specific tumor cell surface receptor or HIV-infected cell surface receptor or receptor carried by other diseased cells and a sequence encoding a protein selected from the proteins, analogs and derivatives of claims 19 and 20 and the p55 TNF-R intracellular domain (p55IC), its 'death domain' (p55DD), the intracellular domain of FAS-R (FAS-IC), or its 'death domain' (FAS-DD), or a biologically active analog or derivative thereof, said protein, when expressed in said tumor, HIV-infected, or other diseased cell being capable of killing said cell; and

(b) infecting said tumor or HIV-infected cells or other diseased cells with said vector of (a).

35. A method for inducing TNF-associated effects in cells or tissues comprising treating said cells with one or more proteins, analogs or derivatives thereof, said one or more proteins being selected from a protein being essentially all of the self-associating intracellular domain of the p55 TNF-R (p55-IC) or portions thereof capable of self-associating and inducing, in a ligand (TNF)-independent manner, said TNF effect in the cells, wherein said treating of the cells comprises introducing into said cells said one or more proteins, analogs or derivatives in a form suitable for intracellular introduction thereof, or introducing into said cells a DNA sequence encoding said one or more proteins, analogs or derivatives in the form of a suitable vector carrying said sequence, said vector being capable of effecting the insertion of said sequence into said cells in a way that said sequence is expressed in said cells.

36. A method according to claim 35, wherein said treating of cells is by transfection of said cells with a recombinant animal virus vector comprising the steps of :

(a) constructing a recombinant animal virus vector carrying a sequence encoding a viral surface protein (ligand) that is capable of binding to a specific cell surface receptor on the surface of said cells to be treated, and a second sequence encoding a protein being the p55-IC, portions thereof, analogs and derivatives of all of the foregoing, said protein when expressed in said cells being capable of self-association and induction of said one or more TNF-associated effects; and

(b) infecting said cells with the vector of (a).

37. A method according to claim 35 or 36, wherein said TNF effect to be induced in said cells is the induction of IL-8 gene expression, said vector carrying a sequence encoding essentially all of said p55-IC, portions thereof, analogs and derivatives of all of the foregoing, which are capable, when expressed in the cells of self-association and signaling for the induction of said IL-8 gene expression.

38. A method according to any one of claims 35-37 for treating tumor cells or virally-infected cells, or for augmenting the antibacterial effect of granulocytes, wherein said viral vector carries a sequence encoding a viral ligand capable of binding a specific cell surface receptor on the surface of said tumor cells, virally-infected cells or granulocytes and a sequence encoding said p55-IC. portions thereof, analogs and derivatives thereof, which when expressed in said tumor, virally-infected or granulocyte cells induces TNF-associated effects leading to the death of these cells.

39. A method according to claim 38 for treating tumor cells, wherein said p55-IC, portions thereof, analogs or derivatives thereof, when expressed in the tumor cells, induce the expression of IL-8 which leads to the killing of said tumor cells by its chemotactic activity which attracts granulocytes and other lymphocytes to the tumor cells resulting in the death of the tumor cells.

40. The intracellular domain of the p55-R (p55-IC), portions, analogs and derivatives of all of the aforegoing for use in the treatment of cells by induction therein of TNF-associated effects.

41. The p55-IC, portions, analogs and derivatives according to claim 40 for use in the treatment of cells by induction therein of IL-8 gene expression.

42. The p55-IC, portions, analogs and derivatives according to claim 41 for use in the treatment of tumor cells by induction therein of IL-8 gene expression resulting in the killing of the tumor cells.

43. A method for modulating the TNF or FAS-R ligand effect on cells comprising applying the ribozyme procedure in which a vector encoding a ribozyme sequence capable of interacting with a cellular mRNA sequence encoding a protein according to claim 19 or 20 or a mRNA sequence encoding p55IC, p55DD, FAS-IC or FAS-DD, is introduced into said cells in a form that permits expression of said ribozyme sequence in said cells, and wherein when said ribozyme sequence is expressed in said cells it interacts with said cellular mRNA sequence and cleaves said mRNA sequence resulting in the inhibition of expression of said protein or said p55IC, p55DD, FAS-IC or FAS-DD in said cells.

44. A method for isolating and identifying proteins, factors or receptors capable of binding to the intracellular domain binding proteins according to claim 19 or 20, comprising applying the procedure of affinity chromatography in which said protein according to claim 19 or 20 is attached to the affinity chromatography matrix, said attached protein is brought into contact with a cell extract and proteins, factors or receptors from cell extract which bound to said attached protein are then eluted, isolated analyzed.

45. A method for isolating and identifying proteins, capable of binding to the intracellular domain binding proteins according to claim 19 or 20, comprising applying the yeast two-hybrid procedure in which a sequence encoding said intracellular domain binding protein is carried by one hybrid vector and sequence from a cDNA or genomic DNA library are carried by the second hybrid vector, the vectors then being used to transform yeast host cells

and the positive transformed cells being isolated, followed by extraction of the said second hybrid vector to obtain a sequence encoding a protein which binds to said intracellular domain binding protein.

46. A pharmaceutical composition for the modulation of the TNF- or FAS-R ligand- effect on cells comprising, as active, ingredient a protein according to claim 19 or 20, or the protein p55IC, p55DD, FAS-IC or FAS-DD, its biologically active fragments, analogs, derivatives or mixtures thereof.

47. A pharmaceutical composition for modulating the TNF- or FAS-R ligand- effect on cells comprising, as active ingredient, a recombinant animal virus vector encoding a protein capable of binding a cell surface receptor and encoding a protein according to claim 19 or 20, or the protein p55IC, p55DD, FAS-IC or FAS-DD, its biologically active fragments or analogs.

48. A pharmaceutical composition for modulating the TNF or FAS-R ligand effect on cells comprising as active ingredient, an oligonucleotide sequence encoding an anti-sense sequence of the sequence according to any one of claims 1-18.

49. A method for isolating and identifying a protein capable of binding to the intracellular domains of TNF-Rs or FAS-R comprising applying the procedure of non-stringent southern hybridization followed by PCR cloning, in which a sequence or parts thereof according to any one of claims 1-18 is used as a probe to bind sequences from a cDNA or genomic DNA library, having at least partial homology thereto, said bound sequences then amplified and cloned by the PCR procedure to yield clones encoding proteins having at least partial; homology to said sequences of claims 1-18.

50. A soluble, oligomeric tumor necrosis factor receptor (TNF-R) comprising at least two self-associated fusion proteins, each fusion protein having (a) at its one end, a TNF binding domain selected from the extracellular domain of a TNF-R, analogs or derivatives thereof, said extracellular domain, analogs or derivatives thereof being incapable of deleterious self-association leading to interference of TNF binding or less than optimal TNF binding, and being able to bind TNF; and (b) at its other end, a self-associating domain selected from (i) essentially all of the intracellular domain of the p55 TNF-R (p55-IC), extending from about amino acid residue 206 to about amino acid residue 426 of the native p55 TNF-R molecule (p55-R); (ii) the death domain of the p55-IC extending from about amino acid residue 328 to about amino acid residue 426 of the native p55-R; (iii) essentially all of the intracellular domain of the Fas/APO1 receptor (Fas-IC); (iv) the death domain of Fas-IC; and (v) analogs, fractions or derivatives of any one of (i)-(iv) being capable of self-association, wherein said at least two self-associated proteins self-associate only at said ends (b), having said ends (a) capable of binding to at least two TNF monomers, each end (a) capable of binding one TNF monomer; and salts and functional derivatives of said soluble, oligomeric TNF-R.

51. A soluble, oligomeric TNF-R according to claim 50 comprising as its at least two ends (a) essentially all of the extracellular domain of the p55 TNF-R (p55-R) extending from about amino acid residue 1 to about amino acid residue 172 of the native p55-R, and as its at least two ends (b), essentially all of said p55-IC.

52. A soluble, oligomeric TNF-R according to claim 50 comprising as its at least two ends (a) essentially all of the extracellular domain of the p55-R extending from about amino acid residue 1 to about amino acid residue 172 of the native p55-R, and as its at least two ends (b) essentially all of said death domain of the p55-IC.

53. A soluble, oligomeric TNF-R according to claim 50 comprising as its at least two ends (a) analogs or derivatives of the extracellular domain of the p55-R, each of said analogs or derivatives being capable of binding one TNF monomer, and being incapable of self-association, and as its at least two ends (b) essentially all of said p55-IC.

54. A soluble, oligomeric TNF-R according to claim 50 comprising as its two ends (a) analogs or derivatives of the extracellular domain of the p55-R, each of said analogs or derivatives being capable of binding one TNF monomer, and being incapable of self-association, and as its at least two ends (b) essentially all of said death domain of p55-IC.

55. A soluble, oligomeric TNF-R according to claim 50 comprising as its at least two ends (a) essentially all of the extracellular domain of the p55-R extending from about amino acid residue 1 to about amino acid residue 172 of the native p55-R, and as its at least two ends, and as its at least two ends (b) essentially all of said Fas-IC.

56. A soluble, oligomeric TNF-R according to claim 50 comprising as its at least two ends (a) essentially all of the extracellular domain of the p55-R extending from about amino acid residue 1 to about amino acid residue 172 of the native p55-R, and as its at least two ends, and as its at leas two ends (b) essentially all of said death domain of Fas-IC.

57. A soluble, oligomeric TNF-R according to claim 50 comprising as its at least two ends (a) analogs or derivatives of the extracellular domain of the p55-R, each of said analogs or derivatives being capable of binding one TNF monomer, and being incapable of self-association, and as its at least two ends (b) essentially all of said Fas-IC.

58. A soluble, oligomeric TNF-R according to claim 50 comprising as its at least two ends (a) analogs or derivatives of the extracellular domain of the p55-R, each of said analogs or derivatives being capable of binding one TNF monomer, and being incapable of self-association, and as its at least two ends (b) essentially all of said death domain of Fas-IC.

59. A process for the production of the soluble, oligomeric TNF-R according to any one of claims 50-58 comprising :

(a) the construction of an expression vector encoding any one of said fusion proteins, the DNA sequence of each of said ends of the fusion protein being obtained from cloned DNA sequences encoding essentially all of said extracellular domain of the TNF-R, analogs or derivatives thereof; and from cloned DNA sequences encoding essentially all of said p55-IC, p55-IC death domain, Fas-IC, Fas-IC death domain, analogs or derivatives of all of the aforegoing, said ends being ligated together to form a fusion protein sequence, and said

fusion protein sequence being inserted into said vector under the control of transcriptional and translational regulatory sequences;

(b) introduction of the vector of (a) into a suitable host cell in which said fusion protein is expressed; and

(c) purification of the fusion protein expressed in said host cells, said fusion protein self-associating prior to, during, or following the purification process to yield a soluble, oligomeric TNF-R.

60. An expression vector comprising a fusion protein sequence encoding said fusion proteins of any one of claims 50-58.

61. A vector according to claim 60 for use in a process according to claim 59.

62. A host cell containing a vector according to claim 60 capable of expressing said fusion protein sequence.

63. A pharmaceutical composition comprising the soluble, oligomeric TNF-R, salts or functional derivatives thereof and mixtures of any of the foregoing, according to any one of claims 50-58, as active ingredient together with a pharmaceutically acceptable carrier.

64. A soluble, oligomeric TNF-R, salts or functional derivatives thereof and mixtures of any of the foregoing, according to any one of claims 50-58, for use in antagonizing the deleterious effect of TNF in mammals, especially in the treatment of conditions wherein an excess of TNF is formed endogenously or is exogenously administered.

65. A soluble, oligomeric TNF-R, salts or functional derivatives thereof and mixtures of any of the foregoing according to any one of claims 50-58, for use in maintaining prolonged beneficial effects of TNF in mammals, when used with TNF exogenously administered.

66. A soluble, oligomeric Fas/APO1 receptor (Fas-R) comprising at least two self-associated fusion proteins, each fusion protein having (a) at its one end, a Fas ligand binding domain selected from the extracellular domain of a Fas-R, analogs or derivatives thereof being incapable of self-associating and being able to bind Fas ligand; and (b) at its other end, a self-associating domain selected from (i) essentially all of the intracellular domain of the p55 TNF-R (p55-IC), extending from about amino acid residue 206 to about amino acid residue 426 of the native p55 TNF-R molecule (p55-R); (ii) the death domain of the p55-IC extending from about amino acid residue 328 to about amino acid residue 426 of the native p55-R; (iii) essentially all of the intracellular domain of the Fas/APO1 receptor (Fas-IC); (iv) the death domain of Fas-IC; and (v) analogs or derivatives of any one of (i)-(iv) being capable of self-association, wherein said at least two self-associated proteins only self-associate at said ends (b) having said ends (a) capable of binding to at least two Fas ligand monomers, each end (a) capable of binding one Fas ligand monomer; and salts and functional derivatives of said soluble, oligomeric Fas-R.

67. A process for the production of the soluble, oligomeric Fas-R according to claim 66 comprising :

(a) the construction of an expression vector encoding any one of said fusion proteins, the DNA sequence of each of said ends of the fusion protein being obtained from cloned DNA

sequences encoding essentially all of said extracellular domain of the Fas-R, analogs or derivatives thereof ; and from cloned DNA sequences encoding essentially all of said p55-IC, p55-IC death domain, Fas-IC, Fas-IC death domain, analogs or derivatives thereof of all the aforegoing, said ends being ligated together to form a fusion protein sequence, and said fusion protein sequence being inserted into said vector under the control of transcriptional and translational regulatory sequences;

(b) introduction of the vector of (a) into a suitable host cell in which said fusion protein is expressed; and

(c) purification of the fusion protein expressed in the host cells, said fusion protein self-associating prior to, during, or following the purification process to yield a soluble, oligomeric Fas-R.

68. An expression vector comprising a fusion protein sequence encoding said fusion proteins of claim 66.

69. A vector according to claim 68 for use in a process according to claim 67.

70. A host cell containing a vector according to claim 68 capable of expressing said fusion protein sequence.

71. A pharmaceutical composition comprising the soluble, oligomeric Fas-R, salts or functional derivatives thereof and mixtures of any of the foregoing, according to claim 66 as active ingredient together with a pharmaceutically acceptable carrier.

72. A soluble, oligomeric Fas-R, salts or functional derivatives thereof and mixtures of any of the foregoing, according to claim 66, for use in antagonizing the deleterious effect of Fas ligand in mammals, in the treatment of conditions wherein an excess of Fas ligand is formed endogenously or is exogenously administered.

73. A soluble, oligomeric receptor having affinity for both TNF and FAS-R ligand (mixed affinity receptor), comprising at least two self-associated fusion proteins, one of which fusion proteins is a TNF-specific TNF-R-derived protein of any one of claims 50-58; and the other fusion protein is a FAS-R ligand-specific FAS-R-derived protein of claim 66.

74. A pharmaceutical composition comprising the mixed affinity receptor according to claim 73.

75. A mixed affinity receptor according to claim 73 for use in antagonizing the deleterious effects of TNF and FAS-R ligand in mammals.

76. A pharmaceutical composition according to claim 46 for treating cells by induction therein of TNF-associated effects, comprising, as active ingredient, p55-IC, portions thereof, analogs and derivatives of all of the aforegoing, and a pharmaceutically acceptable carrier.

77. A pharmaceutical composition according to claim 57 for treating cells by induction therein of TNF-associated effects, comprising, as active ingredient a recombinant animal virus vector encoding p55-IC, portions thereof, analogs and derivatives of all of the aforegoing, and a protein capable of binding a cell surface protein on the cells to be treated.

78. A pharmaceutical composition according to claim 76 or 77 for the treatment of tumor cells, administration of said composition leading to the induction of IL-8 expression, and subsequent killing of the tumor cells.

55.11 clone

FIGURE 1a

(b) 75.3

5'GAATTCGGCACGAGCGGCACGAGGACAGAGTGAGACTCTGTCTCTTAAAATAATAATA
AAAATAAAAATAAAATGTGGGGCCGGGCAAGGTGGCTCATGCCTGTAATCCCAGCACCTT
GGGAGGCTGAGGCAGGAGGATTGCCTAAGCCCAGGAGTTTGACATCAGCCTGGGCAACAT
GGTGAAACCCCATCTCTACAAAAAATGCAAAAATTAGCCAGGTGTGGTGGGTGTGCTCCT
ATAGTCTCAGCTACTCAGGAGGCTGAGGTAGAGGGGATCACCTGAGCCCAGGAAGTTTGG
AGGCTATAGTGAGCTGAAGACCCGCACCATTGCACGCCAGCCTGGAGCAAGAGACNCTGT
CTCCACATAAATAAATAAATAAATAAAAGTGGGGAACTTCTGTGTTAAGTCAGAAGGCAC
CACACAATTTGNATAGCCANCAACCATATTCAATACCCAATCTCTTTATTGCAATATAAG
TATTTGTAAACCCCTACACAAATATTCCCAAGAATAAGTTGGAATATAAATTACTATATC
AATCANCCAATAAAAATAAACACATACAGTATTTATTTCCTGTTGCTCCATATAAAGCTT
TGCTATTTCAATATAAAGCTTACCTAGTATGGTCATTTGAGCCTGAGCAGAGAATATGCC
CAAGCTCGTGCCGAATTC....GGCGNTCTGACTCTCTACTGAACCAAGACTGAATCAGA
GAGACTCGAGTGCNCTTATTTGATTAANCCCAAATTATTGAAACCTNTGATTTTTTCTGG
AGGNGGATGATAAAGATGTGAAAGTGTGATGAACAGTGTGTATCCCTACTCTTGATCCTG
GAACCAGACAAGCAAGAAGCTTTGATTGAAAGCCTATGTGAAAAGCTGGTCAAATTTCGC
GAAGGTGAACGCCCGTCTCTGAGACTGCAGTTGTTAAGCAACCTTTTCCACGGGATGGAT
AAGAATACTCCTGTAAGATACACAGTGTATTGCAGCCTTATTAAAGTGGCAGCATCTTGT
GGGGCCATCCAGTACATCCCAACTGAGCTGGATCAAGTTAGAAAATGGATTTCTGACTGG
AATCTCACCACTGAAAAAAAGCACACCCTTTTAAGACTACTTTATGAGGCACTTGTGGAT
TGTAAGAAGAGTGATGCTGCTTCAAAAGTCATGGTGGAATTGCTCGGAAGTTACACAGAG
GACAATGCTTCCCAGGCTCGAGTTGATGCCCACAGGTGTATTGTACGAGCATTGAAAGAT
CCAAATGCATTTCTTTGTGACCACCTTCTTACTTTAAAACCAGTCAAGTTTGTGGAAGGC
GAGCTTATTCATGATCTTTTAACCATTTGTGTGAGTGCTAAATTGGCATCATATGTCAAG
TTTTATCAGAATAATAAAGACTTCATTGATTCACTTGGCCTGTTACATGAACAGAATATG
GCAAAAATGAGACTACTTACTTTTATGGGAATGGCAGTAGAAAATAAGGAAATTTCTTTT
GACACAATGCAGCAAGAACTTCAGATTGGAGCTGATGATGTTGAAGCATTTGTTATTGAC
GCCGTAAGAACTAAAATGGTCTACTGCAAAATTGATCAGACCCAGAGAAAAGTAGTTGTC
AGTCATAGCACACATCGGACATTTGGAAAACAGCAGTGGCAACAACTGTATGACACACTT
AATGCCTGGAAACAAAATCTGAACAAAGTGAAAAACAGCCTTTTGAGTCTTTCTGATACC
TGAGTTTTTATGCTTATAATTTTTGTTCTTTGAAAAAAAAGCCCTAAATCATAGTAAAAC
ATTATAAACTAAAAAAAAAAAAAAAAAACTCGAG 3'

FIGURE 1b

(c) 75.16

5'GTCCGGTTTACTTTAACTTAGTTTTGCATAGTTCTAGTGCACGTGAAATTGAAAAGTTA
TTTCCCTTTAGCTGTGTTATTATAGAGCAGAAATTCTGTTTTTAAAAATTAGCCTAAGATA
TACTTGTTTTTGTAAAGAAAAATATTTAATGCTTGAACAAAATAAATTGGAGTTGGAGTAG
AATGTAGTTTGAGGAAATTTGCAGCTTCCAATGCCTCTG.....CAGAGGCATTGGAAGCT
GCAAATTTCCTCAAACTACATTCTACTCCAACTCCAATTTATTTTGTTCAAGCATTAAATA
TTTTTCTTTACAAAAACAAGTATATCTTAGGCTAATTTTTAAAAACAGAATTTCTGCTCTA
TAATAACACAGCTAAAGGGAAATAACTTTTCAATTTCACGTGCACTAGAACTATGCAAAAC
TAAGTTAAAGTAAACCGGAC 3'

FIGURE 1c

55.11pep Length: 900 March 10, 1995 19:08 Type: P Check: 9498 ..

```
   1  RVQPQQSPAA APGGTDEKPS GKERRDAGDK DKEQELSEED KQLQDELML
  51  VERLGEKDTS LYRPALEELR RQIRSSTTSM TSVPKPLKFL CPHYGKLKEI
 101  YENMAPGENK RFAADIISVL AMTMSGEREC LKYRLVGSQE ELASWGHEYV
 151  RHLAGEVAKE WQELDDAEKV QREPLLTLVK EIVPYNMAHN AEHEACDLLM
 201  EIEQVDMLEK DIDENAYAKV CLYLTSCVNY VPEPENSALL RCALGVFRKF
 251  TRFPEALRLA LMLNDMELVE DIFTSCKDVV VQKQMAFMLG RHGVFLELSE
 301  DVEEYEDL*TE IMSNVQHNSN FLALARELDI MEPKVPDDIY KTHLENNRFG
 351  GSCSQVDSAR MNLASSFGNG WGNAAFGQDK LLTDDGNKWL YKNKDHGMLS
 401  AAASLGMILL WDVDGGLTQI DKYLYSSEDY IKSGALLACG IVNSGVRNEC
 451  DPALALLSDY VLHNSNTMRL GSIFGLGLAY AGSNREDVLT LLLPVMGDSK
 501  SSMEVAGVTA LACGMIAVGS CNGDVTSTIL QTIMEKSETE LKDTYARWLP
 551  LGLGLNHLGK GEAIEAILAA LEVVSEPFRS FGNTLVDVCA YAGSGNVLKV
 601  QQLLHICSEH FDSKEKEEDK DKKEKKDKDK KEAPADMGAH QGVAVLGIAL
 651  IAMGEEIGAE MALRTFGHLL RYGEPTLRRA VPLALALISV SNPRLNILDT
 701  LSKFSHDADP EVSYNSIFAM GMVGSGTNNA RLAAMLRQLA QYHAKDPNNL
 751  FMVRLAQGLT HLGKGTLTLC PYHSDRQLMS QVAVAGLLTV LVSFLDVRNI
 801  ILGKSHYVLY GLVAAMQPRM LVTFDEELRP LPVSVRVGQA VDVVGQAGKP
 851  KTITGFQTHT TPVLLAHGER AELATEEFLP VTPILEGFVI FGRTPIMISK
```

Underlined: Homolgy to SEN3
BOLD - Basic cluster, nuclear localisation signal
* end of head

FIGURE 1d

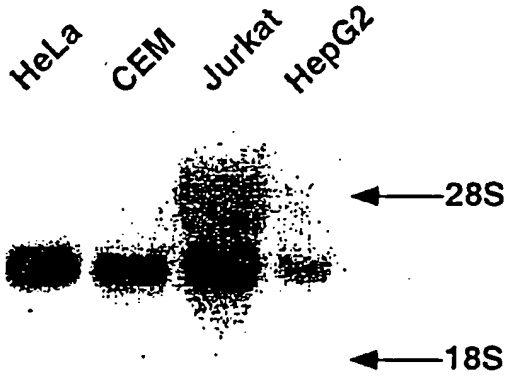
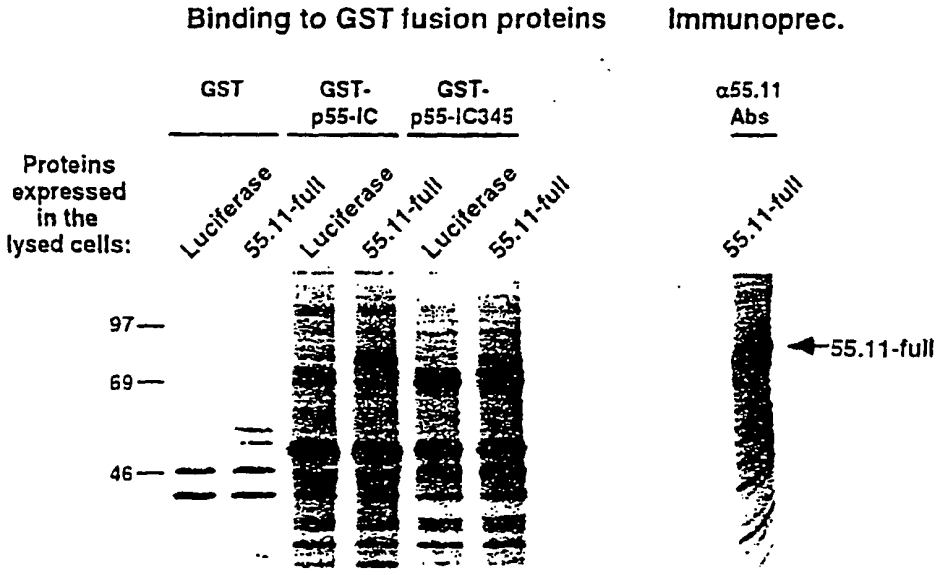


FIGURE 2

A


Binding to GST fusion proteins
Immunoprec.
GST
GST-p55-IC
GST-p55-IC345
α55.11 Abs
Proteins expressed in the lysed cells:
Luciferase
55.11-full
97
69
46
55.11-full

B

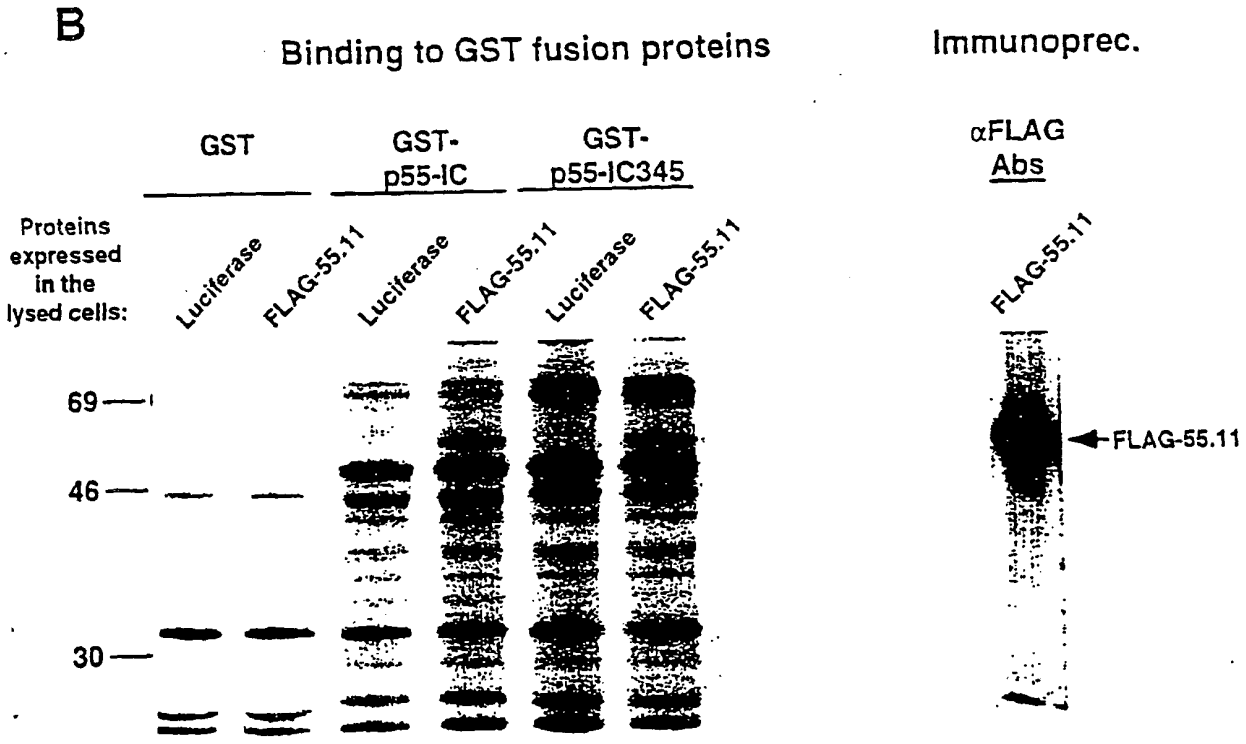

Binding to GST fusion proteins
Immunoprec.
GST
GST-p55-IC
GST-p55-IC345
αFLAG Abs
Proteins expressed in the lysed cells:
Luciferase
FLAG-55.11
69
46
30
FLAG-55.11

FIGURE 3


55.11(human)
YHR027c(yeast)
SEN3(yeast)
A.thaliana(plant)
C.elegans(nematode)

FIGURE 4

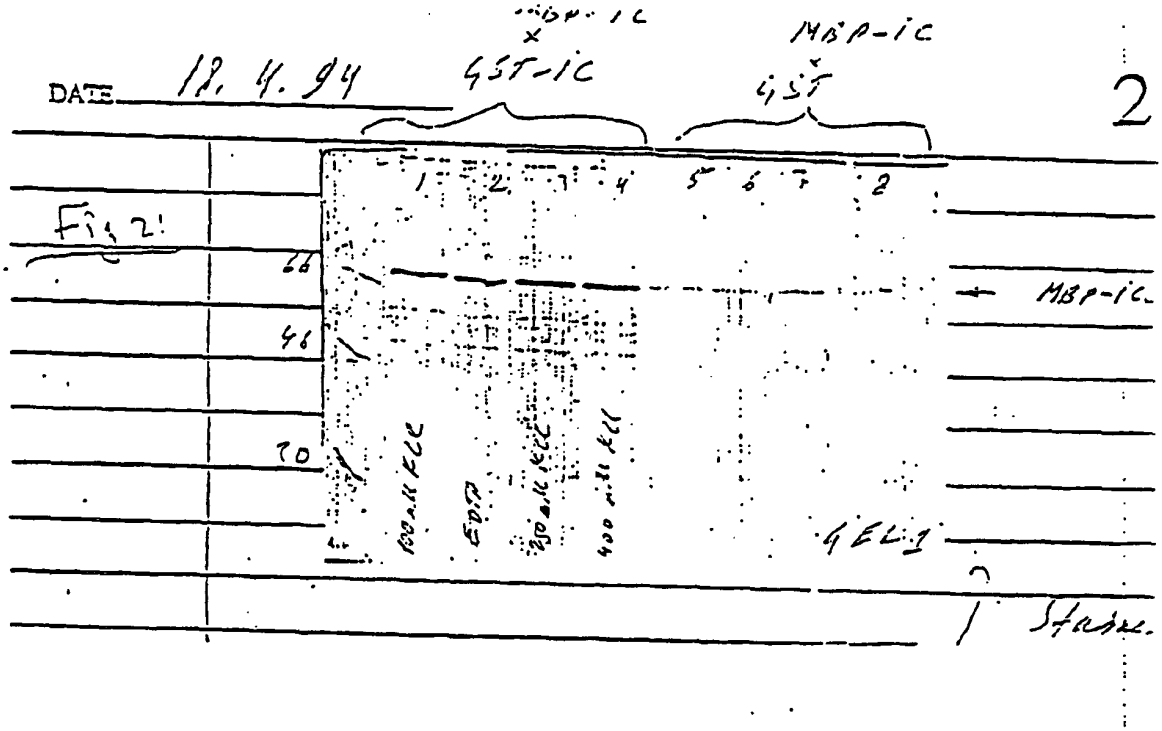


FIGURE 5

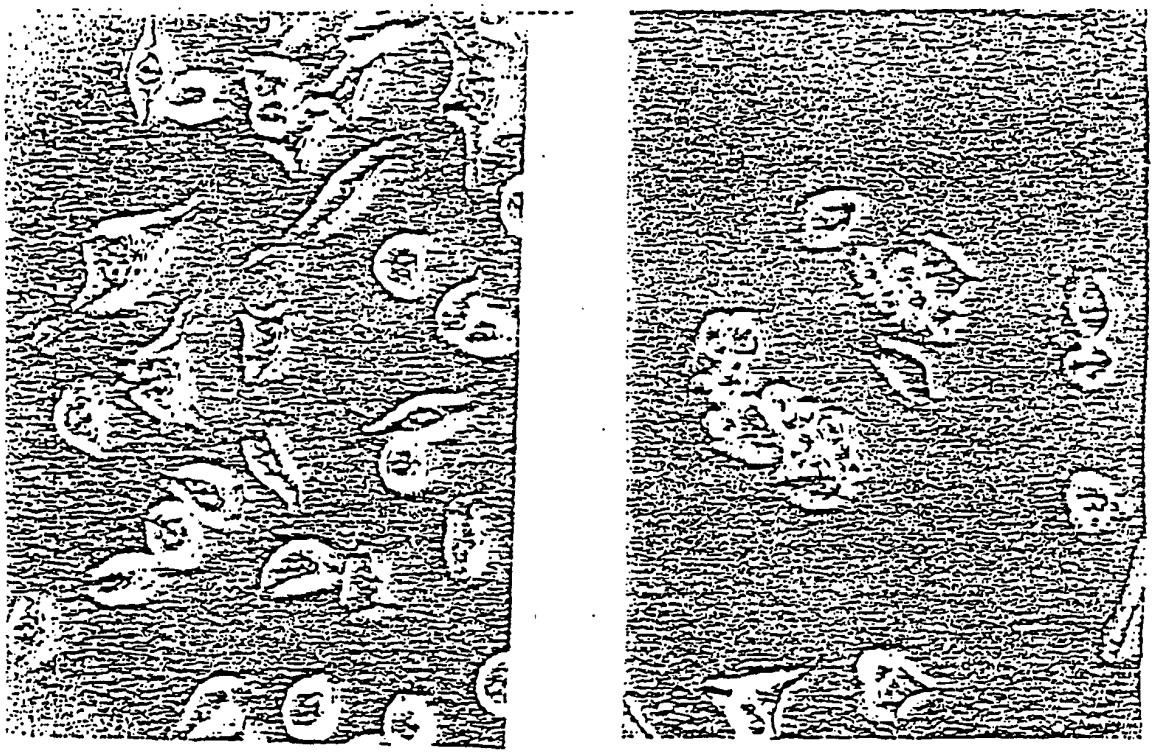


FIGURE 6

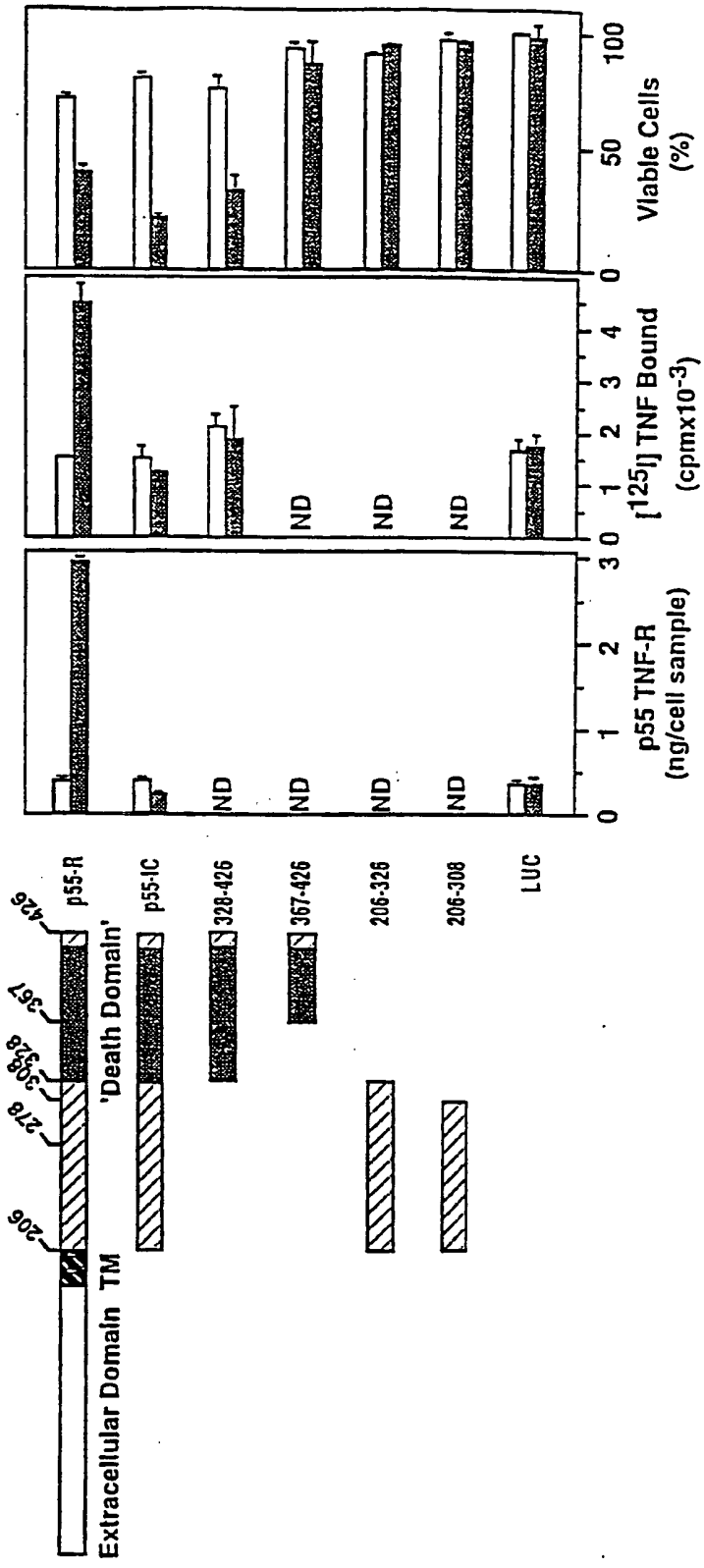
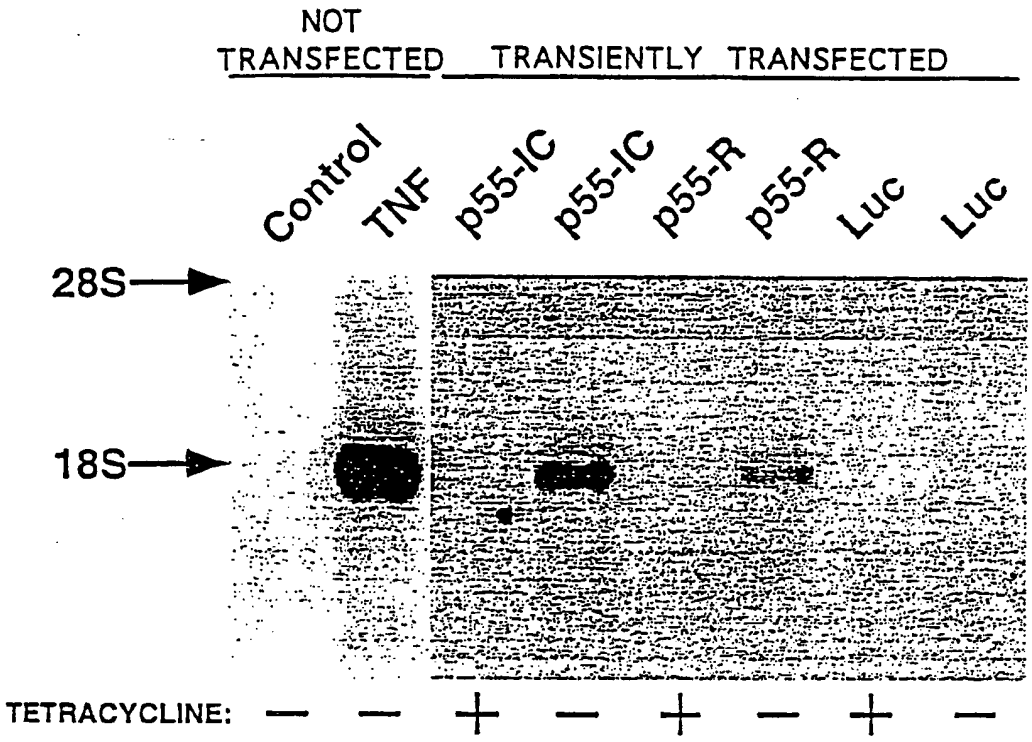

Viable Cells (%)
0
50
100
[125I] TNF Bound (cpmx10-3)
0
1
2
3
4
p55 TNF-R (ng/cell sample)
0
1
2
3
ND
p55-R
p55-IC
328-426
367-426
206-326
206-308
LUC
'Death Domain'
Extracellular Domain
TM
426
367
328
308
278
206

FIGURE 7

A


NOT TRANSFECTED
TRANSIENTLY TRANSFECTED
Control
TNF
p55-IC
p55-IC
p55-R
p55-R
Luc
Luc
28S
18S
TETRACYCLINE: — — + — + — + —

B

FIGURE 8

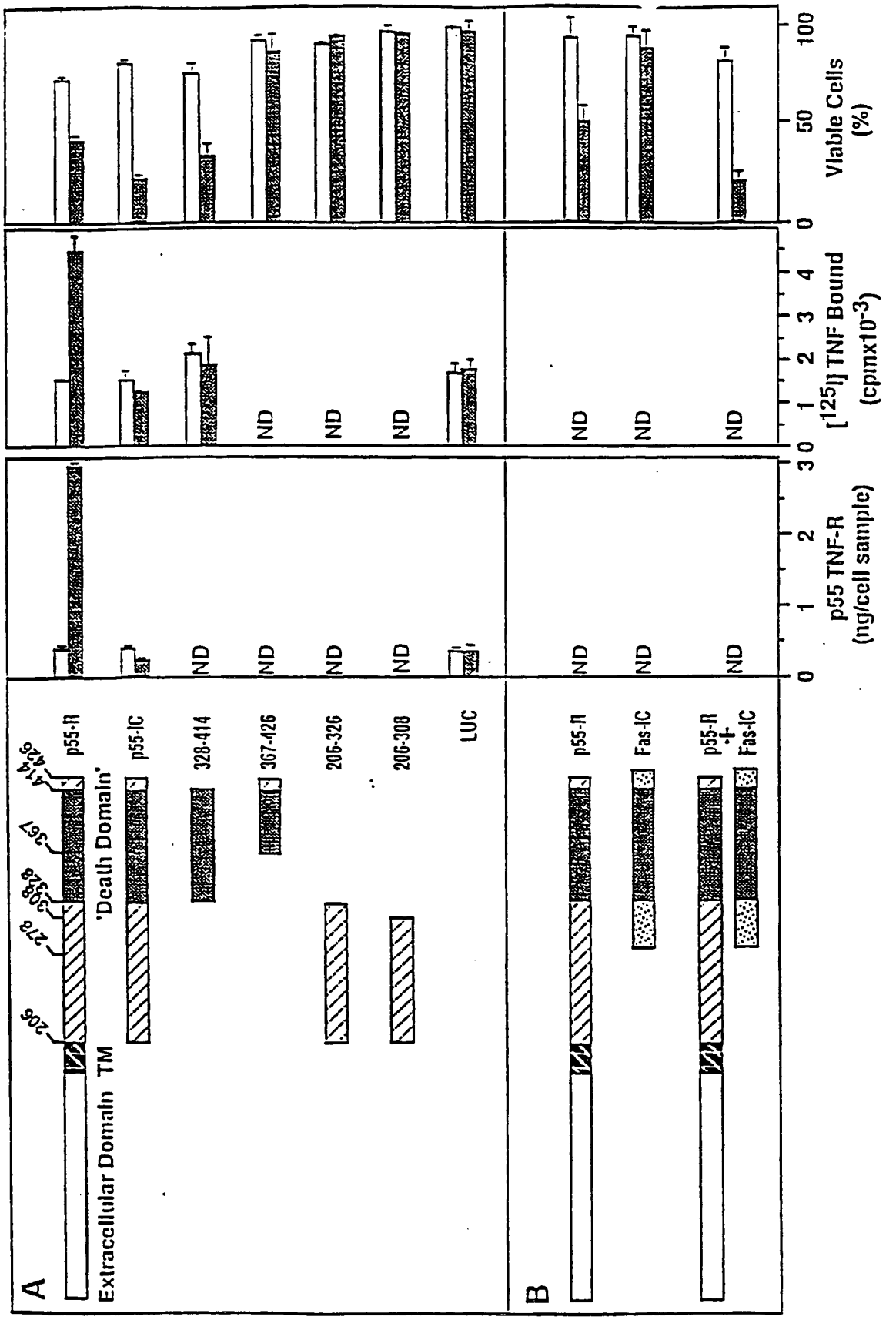

A
B
Extracellular Domain
TM
'Death Domain'
p55-R
p55-IC
328-414
367-426
206-326
206-308
LUC
Fas-IC
p55-R + Fas-IC
206
278
308
328
367
414
426
ND
0
1
2
3
p55 TNF-R (ng/cell sample)
[125I] TNF Bound (cpmx10-3)
0
50
100
Viable Cells (%)

FIGURE 9

F2 clone

FIGURE 10

F9 clone

FIGURE 11

DD11 clone

```
........................................
AATTCGGCACGAGGTTGTGCTGTGGGAAGGGAGAAGGATTTGTAAACCCCGGAGCGAGGTTCTGCTTACCC
GAGGCCGCTGCTGTGCGGAGACCCCCGGGTGAAGCCACCGTCATCATGTCTGACCAGGAGGCAAAACCTTCc
AACTGAGGACTTGGGGGATAAGAAGGAAGGTGAATATATTAAACTCAAAGTCATTGGACAGGATAGCAGTGA
GATTCACTTCAAAGTGAAAATGACAACACATCTCAAGAAACTCAAAGAATCATACTGTCAAAGACAGGGTGT
TCCAATGAATTCACTCAGGTTTCTCTTTGAGGGTCAGAGAATTGCTGATAATCATACTCCAAAAGAACTGGG
AATGGAGAAGAAAGATTGTGATTtGAaGTTTTATCAGGAACAAACGGGGGGTCATTCAACAGcTT.......
..........
```

FIGURE 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05854

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- A	*Proceedings of the National Academy of Sciences USA*, Volume 88, issued April 1991, M. Lewis et al., "Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptor demonstrate one receptor is species specific", pages 2830-2834, especially the abstract and Figure 1.	1-3 ------- 35-37, 40-42, 50-60, 62-68, 70-72, 77
X	EP, A1, 0 510 691 (Nagata et al.) 24 April 1992, especially the abstract and Figures 1-2.	1-3 ------- 50-60, 62-68, 70-72

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 24 AUGUST 1995	Date of mailing of the international search report 29 AUG 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DAVID L. FITZGERALD Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05854

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	*Cell*, Volume 74, issued 10 September 1993, L. A. Tartaglia et al., "A Novel Domain within the 55 kd TNF Receptor Signals Cell Death", pages 845-853.	1-3, 35-37, 40-42, 50-60, 62-68, 70-72, 77
A	*Journal of Biological Chemistry*, Volume 268, Number 15, issued 25 May 1993, N. Itoh et al., "A Novel Protein Domain Required for Apoptosis", pages 10932-10937.	1-3, 35-37, 40-42, 50-60, 62-68, 70-72, 77
A	*Cell*, Volume 73, issued 07 May 1993, D. W. Banner et al., "Crystal Structure of the Soluble Human 55 kd TNF Receptor-Human TNFβ Complex: Implications for TNF Receptor Activation", pages 431-445.	1-3, 35-37, 40-42, 50-60, 62-68, 70-72, 77
A	*Proceedings of the National Academy of Sciences USA*, Volume 90, issued July 1993, L. Van Aelst et al., "Complex formation between RAS and RAF and other protein kinases", pages 6213-6217.	1-3
A,P	*Journal of Biological Chemistry*, Volume 269, Number 31, issued 05 August 1994, B. G. Darnay et al., "Physical and Functional Association of a Serine-Threonine Protein Kinase to the Cytoplasmic Domain of the p80 Form of the Human Tumor Necrosis Factor Receptor in Human Histiocytic Lymphoma U-937 Cells", pages 19687-19690.	1-3
X,P	*Journal of Biological Chemistry*, Volume 269, Number 36, issued 09 September 1994, H. Y. Song et al, "Aggregation of the Intracellular Domain of the Type 1 Tumor Necrosis Factor Receptor Defined by the Two-hybrid System", pages 22492-22495, especially the abstract.	1-3
X,P	*Journal of Biological Chemistry*, Volume 270, Number 8, issued 24 February 1995, H. Y. Song et al., "Identification of a Protein with Homology to hsp90 That Binds the Type 1 Tumor Necrosis Factor Receptor", pages 3574-3581, especially the abstract and Figure 3.	1-3
X,E	*Cell*, Volume 81, issued 19 May 1995, B. Z. Stanger et al., "RIP: A Novel Protein Containing a Death Domain That Interacts with Fas/APO-1 (CD95) in Yeast and Causes Cell Death", pages 513-23, especially the abstract and Figure 2.	1-3
X,E	*Cell*, Volume 81, issued 19 May 1995, pages 505-512, A. M. Chinnaiyan et al., "FADD, a Novel Death Domain-Containing Protein, Interacts with the Death Domain of Fas and Initiates Apoptosis", pages 505-512, especially the abstract and Figure 2.	1-3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05854

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	*Cell*, Volume 78, issued 26 August 1994, M. Rothe et al., "A Novel Family of Putative Signal Transducers Associated with the Cytoplasmic Domain of the 75 kDa Tumor Necrosis Factor Receptor", pages 681-692, especially the abstract, Figure 3, and Figure 8.	1-3
X,E	*Cell*, Volume 81, issued 19 May 1995, H. Hsu et al., "The TNF Receptor 1-Associated Protein TRADD Signals Cell Death and NF-κB Activation", pages 495-504, especially the abstract and Figure 1.	1-3
X,P	*Journal of Biological Chemistry*, Volume 270, Number 14, issued 07 April 1995, M. P. Boldin et al., "A Novel Protein That Interacts with the Death Domain of Fas/APO1 Contains a Sequence Motif Related to the Death Domain", pages 7795-7798, especially the abstract and Figure 4.	1-3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05854

Box I **bservations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-3, as they read on the disclosed inventions of the 55.11, 75.3, and 75.16 clones,
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Please See Extra Sheet.

3. ☒ Claims Nos.: 4-34, 38, 39, 43-49, 61, 69, 73-76, and 78
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05854

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 15/12, 15/62, 15/67, 1/19, 1/21, 5/10; A61K 38/16, 39/395, 48/00; C07K 14/435, 14/715, 16/18, 16/28, 19/00; G01N 33/53, 37/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

536/23.5, 24.5; 435/320.1, 240.2, 252.3, 254.11, 69.1, 69.7, 7.1, 29; 530/350, 395, 387.9, 388.22, 413; 514/2, 44; 424/143.1, 139.1, 93.2

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

536/23.5, 24.5; 435/320.1, 240.2, 252.3, 254.11, 69.1, 69.7, 7.1, 29; 530/350, 395, 387.9, 388.22, 413; 514/2, 44; 424/143.1, 139.1, 93.2

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Medline, SciSearch, Biosis, Embase, CAS, EPO online, Derwent-WPI, USPTO-APS
Search terms: TNF/tumor necrosis factor Receptor; Fas antigen/receptor; death domain; apoptosis; intracellular, cytopasmic; bind?, associat?

BOX I. OBSERVATIONS WHERE CLAIMS WERE FOUND UNSEARCHABLE
2. Where no meaningful search could be carried out, specifically:

Claims 1-3 are generic to a plurality of inventions which do not share a special technical feature which defines an advance over the art according to PCT Rule 13.2, thus to be so linked as to form a single general inventive concept within the meaning of PCT Rule 13.1. The unsearchable inventions are those specific nucleic acids and proteins which are characterized in terms of their sequences, viz., the 55.11, 75.3, and 75.16 clones. The disclosure is not supplemented by a computer-readable Sequence Listing which this Authority requires in order to search sequence databases. See PCT Rule 5.2; PCT Rule 13ter; PCT Administrative Instruction Section 208, as supplemented by Annex C to the PCT Administrative Instructions; and M.P.E.P. § 1823.02. Claims 1-3 accordingly have been searched only insofar as they do not involve the said sequence-dependent inventions.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows.

In view of the art identified in the International Search, this Authority has reconsidered the status of the inventions of the searchable claims with respect to Unity of Invention, and it considers that this application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

This application contains claims directed to more than one species of the first-recited generic invention, to which claims 1-3 are generic. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. The species and the claims readable thereon are as follows:

I. Claims 1-3, 35, 37, and 40-42, as they read on p55-derived DNA, p55-related proteins and pharmaceutical compositions comprising them, and methods of treatment using the protein.

II. Claims 1-3, as they read on p75-derived DNA.

III. Claims 1-3, as they read on Fas receptor-derived DNA.

The following additional inventions are not related as species to the genus of claims 1-3:

IV. Claims 35-37 and 77, as they read on gene-therapeutic methods of treating cells with p55-IC and vectors suitable for use in such methods.

V. Claims 50-60, 62-68, and 70-72, directed to soluble self-associating fusion protein "receptors".

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features, for the following reasons.

The special technical feature of group I which defines an advance over the art is the p55-encoding DNA. This special technical feature is not shared by either of groups II or III, the special technical features of which are, respectively, p75 DNA and FAS receptor DNA, because although the receptors belong to the same superfamily and have some properties in common, they are biologically and structurally discrete products, each DNA defining a separate advance over the art. For like reasons, groups II and III do not share a special technical feature.

The "main invention" of group I comprises the first product (p55-derived DNAs) and its use to make proteins. The invention of group IV embodies a discrete method of using the DNA of group I which in itself defines an advance over the art. The invention of group IV therefore does not correspond to main invention,i.e., group I, as interpreted by this Authority. 37 CFR § 1.475(d).

Group IV does not share a special technical feature with either of groups II or III because it neither employs nor makes the DNAs of the latter groups.

The invention of group V does not share a special technical feature with any of groups I-III. Although the soluble receptors of group V comprise components having a structural similarity to the proteins encoded by the DNAs of groups I-III, such structural similarity per se does not define an advance over the art since the various TNFR DNAs were separately known prior to the instant invention. The unifying special technical feature of group V accordingly resides in novel oligomeric fusion proteins themselves; none of groups I-III shares this special technical feature.

Finally, the invention of group IV does not share a special technical feature with group V because it neither employs nor makes the products of the latter group.

For the above reasons, this Authority considers that the inventions are not so linked in any pairing by a common special technical feature so as to form a single general inventive concept within the meaning of PCT Rule 13.1.